

Appl. No. : 10/063,514  
Filed : May 1, 2002

### REMARKS

Applicants thank the Examiner for his review of the instant application. For the reasons stated below, the rejections of the presently pending claims are respectfully traversed. Claims 6-7, 9, and 11-17 are presented for examination.

#### **Status of the Claims**

Applicants mailed an Amendment After Final Office Action on December 22, 2005. In that Amendment, Applicants canceled Claims 4-5 without prejudice to, or disclaimer of, the subject matter contained therein. Applicants maintain that the cancellation of a claim makes no admission as to its patentability and reserve the right to pursue the subject matter of the cancelled claim in this or any other patent application. Applicants also amended Claim 12 to depend from Claim 6, rather than canceled Claim 4. The listing of the claims above repeat these amendments as it is not known if the previously filed Amendment After Final Office Action was entered.

#### **Rejection Under 35 U.S.C. §101**

The PTO maintains its rejection of pending Claims 6-7, 9, and 11-17 under 35 U.S.C. § 101 as lacking utility for the reasons set forth in the previous Office Actions. The PTO states that the specification discloses that the PRO874 polynucleotide is more highly expressed in normal lung tissue as compared to lung tumor tissue, and that Applicants have asserted the use of the PRO874 polypeptide for diagnosis. However, the PTO rejects this utility because "Applicants have failed to establish the correlation between PRO874 mRNA expression and PRO874 polypeptide expression in normal lung, lung tumor, or any other type of tissue sample." *Office Action* at 3-4.

Applicants incorporate by reference their previously submitted arguments, including those made in the Appeal Brief, and for the reasons of record assert that the specification contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented and therefore must be taken as sufficient to satisfy the utility requirement of 35 U.S.C. § 101. Applicants also submit that for reasons of record, the PTO has not met its burden of providing evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility. However even if the PTO has met its initial burden, Applicants' rebuttal

Appl. No. : 10/063,514  
Filed : May 1, 2002

evidence previously submitted and additional evidence submitted herewith is sufficient to prove that it is **more likely than not** that a person of skill in the art would be convinced, to a **reasonable probability**, that the asserted utility is true. As stated previously, Applicants' evidence need not be direct evidence, so long as there is a reasonable correlation between the evidence and the asserted utility. **The standard is not absolute certainty.**

### Substantial Utility

#### Summary of Applicants' Arguments and the PTO's Response

Applicants' asserted utility rests on the following argument:

1. Applicants have provided reliable evidence that mRNA for the PRO874 polypeptide is expressed at least two-fold higher in normal lung tissue as compared to lung tumor tissue;
2. Applicants assert that it is well-established in the art that a change in the level of mRNA for a particular protein, *e.g.* a decrease, generally leads to a corresponding change in the level of the encoded protein, *e.g.* a decrease;
3. Given the differential expression of the PRO874 mRNA in lung tumors as compared to normal lung tissue, it is more likely than not that the PRO874 polypeptide is also differentially expressed in lung tumors as compared to normal lung tissue, making the claimed polypeptides useful as diagnostic tools, alone or in combination with other diagnostic tools.

Applicants understand the PTO to be making two main arguments in response to Applicants' asserted utility:

1. The PTO challenges the reliability of the evidence reported in Example 18, stating that it cannot be determined if the differential PRO874 mRNA expression is "significant or insignificant, relevant or irrelevant, disease-dependent or disease-independent," citing Hu *et al.* (J. Proteome Res. 2003; 2(4):405-12) and LaBaer (Nature Biotechnol. 2003; 21(9):976-7) for support;
2. The PTO argues that "protein expression levels are not predictable from the mRNA expression levels," citing Haynes *et al.* (Electrophoresis, (1998) 19(11):1862-71), Gygi *et al.* (Mol. and Cell. Bio., (1999) 19(3):1720-30) and Allman *et al.* (Blood, (1996) 87(12):5357-68).

Applicants respectfully submit that in light of all of the evidence, the PTO's arguments are not adequate to support the utility rejection of the claimed invention under 35 U.S.C. § 101.

Appl. No. : 10/063,514  
Filed : May 1, 2002

Applicants have established that the Gene Encoding the PRO874 Polypeptide is Differentially Expressed in Certain Cancers compared to Normal Tissue

Applicants submit that the gene expression data provided in Example 18 of the present application are sufficient to establish that the PRO874 gene is differentially expressed in lung tumor tissue as compared to normal lung tissue, and is therefore useful as a diagnostic tool for cancer, particularly lung cancer.

Applicants previously submitted a first Declaration of J. Christopher Grimaldi, an expert in the field of cancer biology. This declaration explains the importance of the data in Example 18, and how differential gene and protein expression studies are used to differentiate between normal and tumor tissue.

In paragraph 5 of his declaration, Mr. Grimaldi states that the gene expression studies reported in Example 18 of the instant application were made from pooled samples of normal and of tumor tissues. Mr. Grimaldi explains that:

The DNA libraries used in the gene expression studies were made from pooled samples of normal and of tumor tissues. Data from pooled samples is more likely to be accurate than data obtained from a sample from a single individual. That is, the detection of variations in gene expression is likely to represent a more generally relevant condition when pooled samples from normal tissues are compared with pooled samples from tumors in the same tissue type. *First Grimaldi Declaration* at ¶ 5 (emphasis added).

In paragraphs 6 and 7, Mr. Grimaldi explains that the semi-quantitative analysis employed to generate the data of Example 18 is sufficient to determine if a gene is over- or under-expressed in tumor cells compared to corresponding normal tissue. He states that any visually detectable difference seen between two samples is indicative of at least a two-fold difference in cDNA between the tumor tissue and the counterpart normal tissue. Thus, the results of Example 18 reflect at least a two-fold difference between the pooled normal and tumor samples. This detected differential expression in pooled tumor samples compared to pooled normal samples represents a more generally relevant result compared to differential expression detected in samples from a single individual. He also states that the results of such gene expression studies indicate that the genes of interest “can be used to differentiate tumor from normal,” thus establishing their reliability. He further states that if a “difference is detected, this

Appl. No. : 10/063,514  
Filed : May 1, 2002

indicates that the gene and its corresponding polypeptide and antibodies against the polypeptide are useful for diagnostic purposes, to screen samples to differentiate between normal and tumor.”

In response, the PTO relies on Hu, *et al.* and LaBaer, arguing that “reports of mRNA, or protein changes of as little as two-fold are not uncommon, and although some changes of this magnitude turn out to be important, most are attributable to disease-independent differences between samples.” *Office Action* at 8. According to the PTO, this evidence shows that “the skilled artisan would not know if the differences in PRO874 mRNA expression is significant or insignificant, relevant or irrelevant, disease-dependent or disease-independent.” *Id.* at 8.

In addition to the reasons articulated in Applicants’ arguments of record including the Appeal Brief, which Applicant incorporates by reference, the PTO’s reliance on Hu and LaBaer is also misplaced because Applicants are not relying on microarray data as discussed in Hu and LaBaer:

In any microarray experiment, thousands of genes may demonstrate statistically significant expression changes, but only a fraction of these may be relevant to the study. *Hu* at 405, left column, first paragraph (emphasis added).

In the accelerating quest for disease biomarkers, the use of high-throughput technologies, such as DNA microarrays and proteomics experiments, has produced vast datasets identifying thousands of genes whose expression patterns differ in diseased versus normal samples. Although many of these differences may reach statistical significance, they are not always biologically meaningful. *LaBaer* at 976, paragraph bridging middle and right column.

Instead, Applicants are relying on a more accurate and reliable method of assessing changes in mRNA level, namely quantitative PCR analysis. In a recent study by Kuo *et al.*, (*Proteomics* 5(4):894-906 (2005)), the authors used microarray analysis combined with proteomic analysis using two-dimensional gel electrophoresis to examine changes in gene expression in leukemia cell lines. The authors report that “[c]omparison of microarray and proteomic expression profiles showed poor correlation. Use of more reliable and sensitive analyses, such as reverse transcriptase polymerase chain reaction [RT-PCR], Western blotting and functional assays, on several genes and proteins, nonetheless, confirmed that there is indeed good correlation between mRNA and protein expression.” *Kuo et al.* at Abstract (emphasis added) (attached as Exhibit 1). Thus, even if accurate, Hu’s and LaBaer’s statements regarding



Appl. No. : 10/063,514  
Filed : May 1, 2002

microarray studies are not relevant to the instant application which does not rely on microarray data.

Moreover, Hu and LaBaer are silent regarding the reliability of pooled samples, and whether or not differential expression in pooled samples are susceptible to disease-independent differences between samples. The PTO's concern that "it is unknown if the PRO874 transcript differences are disease-dependent or disease-independent" is addressed by the statement in the first Grimaldi Declaration that "DNA libraries used in the gene expression studies were made from pooled samples of normal and of tumor tissues. Data from pooled samples is more likely to be accurate than data obtained from a sample from a single individual." *First Grimaldi Declaration* at ¶ 5. Hu and LaBaer provide no reason to expect that differential expression in pooled samples is attributable to disease-independent differences between samples. Thus, Hu and LaBaer do not provide a basis for doubting Applicants' differential expression data. As such, there is no evidence that one skilled in the art would question whether the differential expression of PRO874 mRNA in pooled samples was disease-dependent or disease-independent.

In conclusion, Applicants submit that the evidence reported in Example 18, supported by the first Grimaldi Declaration, establish that there is at least a two-fold difference in PRO874 mRNA between lung tumor tissue as compared to normal lung tissue. Thus, any challenge to the sufficiency of the data with respect to the utility of the nucleic acid is inappropriate. Therefore, the only issue which remains is whether the data in Example 18 regarding differential expression of the PRO874 mRNA are reasonably correlated with differential expression of the PRO874 polypeptide such that the claimed polypeptides have utility as diagnostic tools as well. As discussed below, even if the PTO has established a reasonable doubt regarding Applicants' assertion that they are reasonably correlated, Applicants' overwhelming rebuttal evidence is more than sufficient to establish that changes in mRNA level lead to corresponding changes in protein level.

*The PTO's Evidence is Not Relevant to Determining Whether a Change in mRNA Level for a Particular Gene leads to a Corresponding Change in the Level of the Encoded Protein*

Applicants turn next to the second portion of their argument in support of their asserted utility – that it is well-established in the art that a change in the level of mRNA encoding a

Appl. No. : 10/063,514  
Filed : May 1, 2002

particular protein generally leads to a corresponding change in the level of the encoded protein; given Applicants' evidence of differential expression of the mRNA for the PRO874 polypeptide in lung tumors, it is likely that the PRO874 polypeptide is also differentially expressed; and proteins differentially expressed in certain tumors have utility as diagnostic tools.

In response to Applicants' assertion, the PTO cites Haynes *et al.* (Electrophoresis, (1998) 19(11):1862-71), Gygi *et al.* (Mol. and Cell. Bio., (1999) 19(3):1720-30) and Allman *et al.* (Blood, (1996) 87(12):5357-68) for support of its argument that "protein levels cannot be accurately predicted from the level of the corresponding mRNA transcript." *Office Action* at 14.

Applicants have previously discussed at length why the Haynes, Gygi and Allman references are not relevant to the issue of whether changes in mRNA level for a particular gene lead to changes in protein level. Applicants incorporate by reference the previous arguments, including those made in their appeal brief, and will not repeat them here.

However, in an attempt to illustrate why references which relate to static global levels of mRNA and protein across different genes are not relevant to this issue, Applicants offer the following illustration and analogy with the understanding that like all illustrations and analogies, they are not perfect and therefore do not represent any admissions or binding statements regarding Applicants' disclosure or invention.

Haynes and Gygi discuss whether there is a correlation between the static level of mRNAs and proteins globally, *i.e.* across different genes. This is equivalent to conducting a hypothetical Experiment 1, where a particular cell type has 100 copies of mRNA for gene X, 200 copies of mRNA for gene Y, and 400 copies of mRNA for gene Z. If there is a global correlation between static mRNA levels and protein levels across genes, the ratio of the amount of proteins X:Y:Z would be approximately 1:2:4. This is essentially what the cited references examined.

In contrast, Applicants are relying on a correlation between changes in mRNA level for a particular gene leading to a corresponding change in the level of the encoded protein. For example, in hypothetical Experiment 2, if gene X has 200 copies of mRNA per cell in condition A (*e.g.* normal), and 100 copies of mRNA for gene X in condition B (*e.g.* tumor), the ratio of the amount of protein X in condition A:B would be approximately 2:1, such that there is a correlation between the change in the level of mRNA and protein for a particular gene.

The PTO argues that because there is no correlation between static levels of mRNA and protein across genes, as illustrated by Experiment 1, one of skill in the art would not expect an increase or decrease in the amount of mRNA for a particular gene to result in a corresponding change in the amount of the encoded protein, as illustrated in Experiment 2. This is simply wrong – there does not need to be a global correlation across genes for there to be a correlation in changes for a particular gene.

For example, Haynes reports that the amount of protein produced by similar levels of mRNA varied by as much as fifty-fold, and that similar amounts of protein were sustained by amounts of mRNA that varied by as much as forty-fold. *Haynes* at 1863, first full paragraph. Based on these results, Haynes concludes that “protein levels cannot be accurately predicted from the level of the corresponding mRNA transcript.” *Id.*

This is analogous to a finding that on one gallon of gas, a hybrid car can travel 70 miles but a large truck can only travel 5 miles, or that to travel 70 miles, a hybrid car requires 1 gallon of gas, but a large truck requires 14 gallons. That is to say, there are many things which affect the fuel efficiency of an automobile. Based on these observations, one could conclude that given the lack of correlation between the amount of gas in an automobile and the distance it travels, one cannot predict how far an automobile will travel based on the amount of gas in the tank.

Even if true, Haynes’ data and conclusions are irrelevant to Applicants’ assertion, which is that increasing or decreasing the amount of mRNA for a particular gene will result in a corresponding increase or decrease in the amount of the encoded protein. This is analogous to increasing or decreasing the amount of gas in an automobile – it will travel farther if you add more gas, and not as far with less. The fact that there are many things which affect fuel efficiency and therefore you cannot predict how far an automobile will travel without knowing if it is a hybrid or a large truck is irrelevant – both a hybrid and a truck travel farther on more gas, and not as far on less.

Applicants emphasize, and the PTO will recognize, that these are simplified illustrations to demonstrate the difference between the two issues being examined. However, these illustrations make clear that even if there is no correlation in the first experiment looking at static levels of mRNA and protein across genes, there can still be a correlation between changes in mRNA and protein for a particular gene as examined in the second experiment. As these

Appl. No. : 10/063,514  
Filed : May 1, 2002

illustrations make clear, the PTO's evidence simply is not relevant to answering the question of whether it is **more likely than not** that a person of skill in the art would be convinced, **to a reasonable probability**, that the asserted utility is true.

*Applicants' Evidence Establishes that a Change in mRNA Level for a Particular Gene lead to Corresponding Change in the Level of the Encoded Protein*

In support of the assertion that changes in mRNA are positively correlated to changes in protein levels, Applicants previously submitted a copy of a second Declaration by J. Christopher Grimaldi, a copy of the declaration of Paul Polakis, Ph.D., excerpts from the Molecular Biology of the Cell, a leading textbook in the field (Bruce Alberts, *et al.*, Molecular Biology of the Cell (3<sup>rd</sup> ed. 1994) and (4<sup>th</sup> ed. 2002), excerpts from the textbook, Genes VI, (Benjamin Lewin, Genes VI (1997)), a reference by Zhigang *et al.*, World Journal of Surgical Oncology 2:13, 2004, and a reference by Meric *et al.*, Molecular Cancer Therapeutics, vol. 1, 971-979 (2002). The details of the teachings of these declarations and references, and how they support Applicants' asserted utility, are of record and will not be repeated here.

Applicants submit herewith a copy of a second Declaration by Dr. Polakis (attached as Exhibit 2) that presents evidentiary data in Exhibit B. Exhibit B of the Declaration identifies 28 gene transcripts out of 31 gene transcripts (i.e., greater than 90%) that showed good correlation between tumor mRNA and tumor protein levels. As Dr. Polakis' second Declaration says "[a]s such, in the cases where we have been able to quantitatively measure both (i) mRNA and (ii) protein levels in both (i) tumor tissue and (ii) normal tissue, we have observed that in the vast majority of cases, there is a very strong correlation between increases in mRNA expression and increases in the level of protein encoded by that mRNA." Accordingly, Dr. Polakis has provided the facts to enable the PTO to draw independent conclusions.

The case law has clearly established that in considering affidavit evidence, the PTO must consider all of the evidence of record anew. *See in re Rinehart*, 531 F.2d 1084, 189 USPQ 143 (C.C.P.A. 1976); *In re Piasecki*, 745 F.2d. 1015, 226 USPQ 881 (Fed. Cir. 1985). "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument." *In re Alton*, 37 U.S.P.Q.2d 1578, 1584 (Fed. Cir. 1996), *quoting In*

Appl. No. : 10/063,514  
Filed : May 1, 2002

*re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992). Furthermore, the Federal Court of Appeals held in *In re Alton*, “We are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner.” *Id.* at 1583. Applicants also respectfully draw the PTO’s attention to the Utility Examination Guidelines which state, “Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.” 66 Fed. Reg. 1098, Part IIB (2001).

In addition to the supporting references previously submitted by Applicants, Applicants submit the following references to further support the assertion that changes in mRNA levels generally lead to corresponding changes in the level of the encoded polypeptide.

In a comprehensive study by Orntoft *et al.* (Mol. Cell. Proteomics. 2002; 1(1):37-45) (previously submitted with IDS, attached hereto as Exhibit 3), the authors examined gene amplification, mRNA expression level, and protein expression in pairs of non-invasive and invasive human bladder tumors. *Id.* at Abstract. The authors examined 40 well resolved abundant known proteins, and found that “[i]n general there was a highly significant correlation ( $p < 0.005$ ) between mRNA and protein alterations. Only one gene showed disagreement between transcript alteration and protein alteration.” *Id.* at 42, col. 2. The alternations in mRNA and protein included both increases and decreases. *Id.* at 43, Table II. Clearly, a correlation in 39 of 40 genes examined supports Applicants’ assertion that changes in mRNA level generally lead to corresponding changes in protein level.

In a study by Wang *et al.* (Urol. Res. 2000; 28(5):308-15) (abstract attached as Exhibit 4) the authors report that down-regulation of E-cadherin protein has been shown in various human tumors. *Id.* at Abstract. In the reported study, the authors examined the expression of cadherins and associated catenins at the mRNA level in paired tumor and nonneoplastic primary prostate cultures. They report that “[s]ix of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of alpha-catenin and beta-catenin mRNA were also observed.” *Id.* As Applicants’ assertion would predict, the authors state that the mRNA measures showed “good correlation” with the results from protein measures. The authors conclude by stating that “this paper presents a coordinated down-

regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied.” *Id.*

In a more recent study by Munaut *et al.* (Int. J. Cancer. 2003; 106(6):848-55) (abstract attached as Exhibit 5) the authors report that vascular endothelial growth factor (VEGF) is expressed in 64-95% of glioblastomas (GBMs), and that VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. *Id.* at Abstract. The authors explain that infiltrating tumor cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). In the present study, the authors “used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels.” *Id.* Thus, the results support Applicants’ assertion that changes in mRNA level lead to corresponding changes in protein level.

In another recent study, Hui *et al.* (Leuk. Lymphoma. 2003; 44(8):1385-94 (abstract attached as Exhibit 6) used real-time quantitative PCR and immunohistochemistry to evaluate cyclin D1 mRNA and protein expression levels in mantle cell lymphoma (MCL). *Id.* at Abstract. The authors report that seven of nine cases of possible MCL showed overexpression of cyclin D1 mRNA, while two cases showed no cyclin D1 mRNA increase. *Id.* Similarly, “[s]ix of the seven cyclin D1 mRNA overexpressing cases showed increased cyclin D1 protein on tissue array immunohistochemistry; one was technically suboptimal.” *Id.* The authors conclude that the study “demonstrates good correlation and comparability between measure of cyclin D1 mRNA ... and cyclin D1 protein.” *Id.* Thus, this reference supports Applicants’ assertion.

In a recent study by Khal *et al.* (Int. J. Biochem. Cell Biol. 2005; 37(10):2196-206) (abstract attached as Exhibit 7) the authors report that atrophy of skeletal muscle is common in patients with cancer and results in increased morbidity and mortality. *Id.* at Abstract. To further understand the underlying mechanism, the authors studied the expression of the ubiquitin-proteasome pathway in cancer patient muscle using a competitive RT-PCR to measure expression of mRNA for proteasome subunits C2 and C5, while protein expression was

Appl. No. : 10/063,514  
Filed : May 1, 2002

determined by western blotting. "Overall, both C2 and C5 gene expression was increased by about three-fold in skeletal muscle of cachectic cancer patients (average weight loss 14.5+/-2.5%), compared with that in patients without weight loss, with or without cancer. ... There was a good correlation between expression of proteasome 20Salpha subunits, detected by western blotting, and C2 and C5 mRNA, showing that increased gene expression resulted in increased protein synthesis." These findings support Applicants' assertion that changes in mRNA level lead to changes in protein level.

Maruyama *et al.* (Am. J. Patho. 1999; 155(3):815-22) (abstract attached as Exhibit 8) investigated the expression of three Id proteins (Id-1, Id-2 and Id-3) in normal pancreas, in pancreatic cancer and in chronic pancreatitis (CP). The authors report that pancreatic cancer cell lines frequently coexpressed all three Ids, "exhibiting good correlation between Id mRNA and protein levels." *Id.* at Abstract. In addition, the authors teach that all three Id mRNA levels were expressed at high levels in pancreatic cancer samples compared to normal or CP samples. At the protein level, Id-1 and Id-2 staining was faint in normal tissue, while Id-3 ranged from weak to strong. In contrast, in the cancer tissues "many of the cancer cells exhibited abundant Id-1, Id-2, and Id-3 immunoreactivity," and Id-1 and Id-2 protein was increased significantly in the cancer cells by comparison to the respective controls, mirroring the overexpression at the mRNA level. Thus, the authors report that in both cell lines and tissue samples, increased mRNA levels leads to an increase in protein overexpression, supporting Applicants' assertion.

Support for Applicants' assertion is also found in an article by Caberlotto *et al.* (Neurosci. Lett. 1999; 256(3):191-4) (abstract attached as Exhibit 9). In a previous study, the authors investigated alterations of neuropeptide Y (NPY) mRNA expression in the Flinders Sensitive Line rats (FSL), an animal model of depression. *Id.* at Abstract. The authors reported that in the current study, that NPY-like immunoreactivity (NPY-LI) was decreased in the hippocampal CA region, and increased in the arcuate nucleus, and that fluoxetine treatment elevated NPY-LI in the arcuate and anterior cingulate cortex. The authors state that "[t]he results demonstrate a good correlation between NPY peptide and mRNA expression." Thus, increases and decreases in mRNA levels were reflected in corresponding changes in protein level.

Mizrachi and Shemesh (Biol. Reprod. 1999; 61(3):776-84) (abstract attached as Exhibit 10) investigated their hypothesis that FSH regulates the bovine cervical prostaglandin E(2)

Appl. No. : 10/063,514  
Filed : May 1, 2002

(PGE(2)) synthesis that is known to be associated with cervical relaxation and opening at the time of estrus. *Id.* at Abstract. Cervical tissue from pre-estrous/estrous, luteal, and postovulatory cows were examined for the presence of bovine (b) FSH receptor (R) and its corresponding mRNA. The authors report that bFSHR mRNA in the cervix was maximal during pre-estrus/estrus, and that the level of FSHR protein was significantly higher in pre-estrous/estrous cervix than in other cervical tissues. *Id.* The authors state that “[t]here was a good correlation between the 75-kDa protein expression and its corresponding transcript of 2.55 kb throughout the estrous cycle as described by Northern blot analysis as well as RT-PCR.” *Id.* Thus, changes in the level of mRNA for bFSHR led to corresponding changes in FSHR protein levels, a result which supports Applicants’ assertion.

In a study by Stein *et al.* (J. Urol. 2000; 164(3 Pt 2):1026-30) (abstract attached as Exhibit 11), the authors studied the role of the regulation of calcium ion homeostasis in smooth muscle contractility. *Id.* at Abstract. The authors investigated the correlation between sarcoplasmic endoplasmic reticulum, calcium, magnesium, adenosine triphosphatase (SERCA) protein and gene expression, and the contractile properties in the same bladder. Partial bladder outlet obstructions were created in adult New Zealand white rabbits, which were divided into control, sham operated and obstructed groups. Stein *et al.* report that “[t]he relative intensities of signals for the Western [protein] and Northern [mRNA] blots demonstrated a strong correlation between protein and gene expression. ... The loss of SERCA protein expression is mediated by down-regulation in gene expression in the same bladder.” *Id.* This report supports Applicants’ assertion that changes in mRNA level, e.g. a decrease, lead to a corresponding change in the level of the encoded protein, e.g. a decrease.

In an article by Gou and Xie (Zhonghua Jie He He Hu Xi Za Zhi. 2002; 25(6):337-40) (abstract attached as Exhibit 12) the authors investigated the expression of macrophage migration inhibitory factor (MIF) in human acute respiratory distress syndrome(ARDS) by examining the expression of MIF mRNA and protein in lung tissue in ARDS and normal persons. *Id.* at Abstract. The authors report “undetectable or weak MIF mRNA and protein expression in normal lungs. In contrast, there was marked upregulation of MIF mRNA and protein expression in the ARDS lungs.” *Id.* This is consistent with Applicants’ assertion that a change in mRNA



Appl. No. : 10/063,514  
Filed : May 1, 2002

for a particular gene, e.g. an increase, generally leads to a corresponding change in the level of protein expression, e.g. an increase.

These studies are representative of numerous published studies which support Applicants' assertion that changes in mRNA level generally lead to corresponding changes in the level of the expressed protein. Applicants submit herewith an additional 70 references (abstracts attached as Exhibit 13) which support Applicants' assertion.

In addition to these supporting references, Applicants also submit herewith additional references which offer indirect support of Applicants' asserted utility. As discussed in detail above, Applicants have challenged the relevance of references such as Haynes *et al.*, and Gygi *et al.* which do not attempt to examine the correlation between a change in mRNA level and a change in the level of the corresponding protein level for a particular gene. Because the PTO continues to rely on these references, Applicants are submitting references which report results that are contrary to the PTO's cited references and offer indirect support for Applicants' asserted utility.

For example, in an article by Futcher *et al.* (Mol. Cell Biol. 1999; 19(11):7357-68) (abstract attached as Exhibit 14) the authors conducted a study of mRNA and protein expression in yeast which was nearly identical to the one conducted by Gygi *et al.* and reported in Haynes *et al.* Contrary to the results of the earlier study by Gygi, Futcher *et al.* report "a good correlation between protein abundance, mRNA abundance, and codon bias." *Id.* at Abstract.

In a study which is more closely related to Applicants' asserted utility, Godbout *et al.* (J. Biol. Chem. 1998; 273(33):21161-8) (abstract attached as Exhibit 15) studied the DEAD box gene, DDX1, in retinoblastoma and neuroblastoma tumor cell lines. The authors report that "there is a good correlation with DDX1 gene copy number, DDX1 transcript levels, and DDX1 protein levels in all cell lines studied." *Id.* Thus, in these cancer cell lines, DDX1 mRNA and protein levels are correlated.

Similarly, in an article by Papotti *et al.* (Virchows Arch. 2002; 440(5):461-75) (abstract attached as Exhibit 16) the authors examined the expression of three somatostatin receptors (SSTR) at the mRNA and protein level in forty-six tumors. *Id.* at Abstract. The authors report a "good correlation between RT-PCR [mRNA level] and IHC [protein level] data on SSTR types 2, 3, and 5." *Id.*

Appl. No. : 10/063,514  
Filed : May 1, 2002

Van der Wilt *et al.* (Eur. J. Cancer. 2003; 39(5):691-7) (abstract attached as Exhibit 17) studied deoxycytidine kinase (dCK) in seven cell lines, sixteen acute myeloid leukemia samples, ten human liver samples, and eleven human liver metastases of colorectal cancer origin. *Id.* at Abstract. The authors report that “enzyme activity and protein expression levels of dCK in cell lines were closely related to the mRNA expression levels” and that there was a “good correlation between the different dCK measurements in malignant cells and tumors.” *Id.*

Grenback *et al.* (Regul. Pept. 2004; 117(2):127-39) (abstract attached as Exhibit 18) studied the level of galanin in human pituitary adenomas using a specific radioimmunoassay. *Id.* at Abstract. The authors report that “[i]n the tumors analyzed with in situ hybridization there was a good correlation between galanin peptide levels and galanin mRNA expression.” *Id.*

Similarly, Shen *et al.* (Blood. 2004; 104(9):2936-9) (abstract attached as Exhibit 19) examined the level of B-cell lymphoma 2 (BCL2) protein expression in germinal center (GC) B-cells and diffuse large B-cell lymphoma (DLBCL). *Id.* at Abstract. The authors report that “GC cells had low expression commensurate with the low protein expression level” and that in DLBCL the level of BCL2 mRNA and protein expression showed “in general, a good correlation.” *Id.*

Likewise, in an article by Fu *et al.* (Blood 2005; 106(13):4315-21) (abstract attached as Exhibit 20) the authors report that six mantle cell lymphomas studied “expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases).” *Id.* at Abstract. “There was a good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis.” *Id.*

These examples are only a few of the many references Applicants could cite in rebuttal to the PTO’s arguments. Applicants submit herewith 26 additional references (abstracts attached as Exhibit 21) which also support Applicants’ assertion in that the references report a correlation between the level of mRNA and corresponding protein, contrary to the assertion of the PTO that mRNA and protein levels are not correlated.

In summary, Applicants submit herewith a total of 113 references and an additional expert Declaration in addition to the declarations and references already of record, which support Applicants’ asserted utility, either directly or indirectly. This evidence supports the assertion that in general, a change in mRNA expression level for a particular gene leads to a corresponding

change in the level of expression of the encoded protein. As Applicants have previously acknowledged, the correlation between changes in mRNA level and protein level is not exact, and there are exceptions (*see, e.g.*, abstracts attached as Exhibit 22). However, Applicants remind the PTO that the asserted utility does not have to be established to a statistical certainty, or beyond a reasonable doubt. *See M.P.E.P.* at § 2107.02, part VII (2004). Therefore, the fact that there are exceptions to the correlation between changes in mRNA and changes in protein does not provide a proper basis for rejecting Applicants' asserted utility. Applicants submit that considering the evidence as a whole, with the overwhelming majority of the evidence supporting Applicants' asserted utility, a person of skill in the art would conclude that Applicants' asserted utility is "more likely than not true." *Id.*

In conclusion, Applicants submit that they have offered sufficient evidence to establish that it is more likely than not that one of skill in the art would believe that because the PRO874 mRNA is differentially expressed in lung tumor tissue as compared to normal lung tissue, the PRO874 polypeptide will likewise be differentially expressed in lung tumors. This differential expression of the PRO874 polypeptide makes the claimed polypeptides useful as diagnostic tools for cancer, particularly lung cancer.

### **Specific Utility**

#### **The Asserted Substantial Utilities are Specific to the Claimed Polypeptides**

Applicants next address the PTO's assertion that the asserted utilities are not specific to the claimed polypeptides related to PRO874. Applicants respectfully disagree.

Specific utility is defined as utility which is "specific to the subject matter claimed," in contrast to "a general utility that would be applicable to the broad class of the invention." *M.P.E.P.* § 2107.01 I. Applicants submit that the evidence of differential expression of the PRO874 gene and polypeptide in certain types of tumor cells, along with the declarations and references discussed above, provide a specific utility for the claimed polypeptides.

As discussed above, there are significant data which show that the gene for the PRO874 polypeptide is differentially expressed by at least two-fold in lung tumor tissue as compared to normal lung tissue. These data are strong evidence that the PRO874 gene and polypeptide are associated with lung tumors. Thus, contrary to the assertions of the PTO, Applicants submit that

Appl. No. : 10/063,514  
Filed : May 1, 2002

they have provided evidence associating the PRO874 gene and polypeptide with a specific disease. The asserted utility for the claimed polypeptides as diagnostic tools for cancer, particularly lung tumors, is a specific utility – it is not a general utility that would apply to the broad class of polypeptides.

#### **Utility – Conclusion**

Applicants remind the PTO that the evidence supporting utility does not need to be direct evidence, nor does it need to provide an exact correlation between the submitted evidence and the asserted utility. Instead, evidence which is “reasonably” correlated with the asserted utility is sufficient. *See Fujikawa v. Wattanasin*, 93 F.3d 1559, 1565, 39 U.S.P.Q. 2d 1895 (Fed. Cir. 1996) (“a ‘rigorous correlation’ need not be shown in order to establish practical utility; ‘reasonable correlation’ suffices”); *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739 (Fed. Cir. 1985) (same); *Nelson v. Bowler*, 626 F.2d 853, 857, 206 U.S.P.Q. 881 (C.C.P.A. 1980) (same). In addition, utility need only be shown to be “more likely than not true,” not to a statistical certainty. *M.P.E.P.* at § 2107.02, part VII (2004). Considering the evidence as a whole in light of the relevant standards for establishing utility, Applicants have established at least one specific, substantial, and credible utility. In view of the above, Applicants respectfully request that the PTO reconsider and withdraw the utility rejection under 35 U.S.C. §101.

#### **Rejections under 35 U.S.C. § 112, first paragraph – Enablement**

The PTO also maintains its rejection of pending Claims 6-7, 9, and 11-17 under 35 U.S.C. § 112, first paragraph. Specifically, the PTO asserts that because the claimed invention is not supported by either a specific or substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention. *Office Action* at 20. In addition, the PTO asserts that the enablement would not be commensurate in scope with pending Claims 12-17. *Office Action* at 21-23.

Appl. No. : 10/063,514  
Filed : May 1, 2002

*The PTO has Failed to Establish a Reasonable Basis to Question the Enablement of the Pending Claims*

As an initial matter, Applicants submit that in the discussion of the 35 U.S.C. § 101 rejection above, Applicants have established a substantial, specific, and credible utility for the claimed polypeptides. To the extent that the enablement rejection is based on a lack of utility, Applicants respectfully request that the PTO reconsider and withdraw the enablement rejection under 35 U.S.C. §112.

Applicants note that Claims 4 and 5 have been canceled, and Claim 12, which depended from Claim 4, has been amended to depend from Claim 6. Claims 12-13 as dependent from Claim 6 do not recite percent amino acid sequence identity as a limitation, nor do they recite any limitation regarding underexpression in lung tumors. These claims are directed to fusion peptides of the disclosed sequence. Other than the enablement rejection based on lack of utility, the PTO has not offered any arguments regarding a lack of enablement for Claim 6. Therefore, the PTO's scope of enablement rejection of pending Claims 12-13 is moot.

With respect to Claims 14-17, which recite the limitation "wherein said isolated polypeptide or a fragment thereof can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO:10 in lung tissue samples," the PTO states that "[t]hese claims encompass any and all antigenically cross-reactive polypeptides possessing the recited percent identity, regardless of their biological activity." *Id.* at 22. The PTO continues, arguing:

If mere antigenic cross-reactivity were the test for enablement under § 112, Applicants could obtain patent rights that may confer power to block off whole areas of scientific development related to the biological activity of the polypeptide, for which Applicants have not provided any disclosure. It is entirely unclear why the disclosure of a single polypeptide, i.e., PRO874 (SEQ ID NO: 10), which is ideally suited to the making of antibodies to itself, would enable any and all antigenically cross-reactive polypeptides possessing the recited percent identity and possessing unknown and undisclosed biological activities, when the specification provides no disclosure of any biological activity. Therefore, the scope of enablement provided to the skilled artisan by the disclosure is not commensurate with the scope of protection sought by the claims. *Id.* at 22-23.

The standard for determining whether the specification meets the enablement requirement is to be evaluated based on whether or not the experimentation needed for one skilled in the art to

Appl. No. : 10/063,514  
Filed : May 1, 2002

practice the invention would be undue. *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916); *In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988); *M.P.E.P.* § 2164.01. Applicants submit that in view of the requirements of enablement under 35 U.S.C. §112, first paragraph, the PTO has failed to establish a *prima facie* basis for rejecting Claims 14-17 as lacking enablement. The PTO's statements fail to establish a reasonable basis to question the enablement provided for the claimed invention. See *M.P.E.P.* § 2164.04.

It is incumbent for the PTO "to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *Id.* (quoting *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971)). This can be done "by making specific findings of fact, supported by the evidence, and then drawing conclusions based on these findings of fact." *Id.* The PTO has failed to make any specific findings of fact, or back up its assertions with any acceptable evidence or reasoning.

In the present case, the PTO reasons that "[i]f mere antigenic cross-reactivity were the test for enablement under § 112, Applicants could obtain patent rights that may confer power to block off whole areas of scientific development related to the biological activity of the polypeptide, for which Applicants have not provided any disclosure." This is not the test for enablement.

The M.P.E.P. states that "if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention." *M.P.E.P.* § 2164.01(c) (emphasis added). As described above, the specification adequately discloses how to make and use the polypeptides of Claims 14-17. The PTO has not alleged that undue experimentation would be required to practice the claimed invention, only that the claimed scope of the invention is too broad. Accordingly, it remains unquestioned that the claimed polypeptides have an enabled use.

Asserting that "[i]t is entirely unclear why the disclosure of a single polypeptide, ... would enable any and all antigenically cross-reactive polypeptides possessing the recited percent identity and possessing unknown and undisclosed biological activities, when the specification provides no disclosure of any biological activity," is also not a reason to reject the claimed polypeptides as lacking enablement. The subject matter of Claims 14-17 relates to isolated polypeptides with at least 95% identity to the disclosed polypeptides wherein the claimed

Appl. No. : 10/063,514  
Filed : May 1, 2002

polypeptides can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO: 10 in lung tissue samples. The PTO has not offered any explanation of how the failure to disclose “any biological activity” results in one of skill in the art having to resort to undue experimentation to practice the claimed invention. Disclosure of a “biological activity” is not required for one of skill in the art to either make or use the claimed polypeptides.

Further, “[t]he presence of only one example should never be the sole reason for rejecting claims as being broader than the enabling disclosure, even though it is a factor to be considered along with all the other factors. To make a valid rejection, one must evaluate all the facts and evidence and state why one would not expect to be able to extrapolate that one example across the entire scope of the claims.” *M.P.E.P.* § 2164.02. The PTO provides no other basis for rejection of the Claims 14-17 aside from pointing to “disclosure of a single polypeptide.” Accordingly, pointing to the disclosure of a single polypeptide, absent any other evidence, cannot support *prima facie* rejection of lack of enablement of the claimed polypeptides.

Notwithstanding the failure of the PTO to provide sufficient evidence to support a *prima facie* rejection of Claims 14-17, the specification teaches in detail how to make the claimed polypeptides, including variants thereof, and antibodies which specifically bind PRO874. *See, e.g.*, ¶¶ [0283]-[0315]; [0256]-[0271]; [0361]-[0379]; and Examples 6-10 (¶¶ [0453]-[0499]). In addition, the specification discloses that antibodies to claimed polypeptides can be used in diagnostic assays to detect the expression of PRO874 in specific types of tissue. *See e.g., Specification* at [0407].

Thus, there is significant guidance how to make and use the claimed polypeptides. In addition, as the disclosure and references cited in the specification make clear, the production of polypeptides, polypeptide variants, and specific antibodies is a predictable and well established aspect of the biological sciences. *See, e.g., In re Wands*, 858 F.2d 731, 8 U.S.P.Q. 2d 1400 (Fed. Cir. 1988) (reversing the Board’s decision of non-enablement and holding that as of 1980, undue experimentation was not required to make high-affinity monoclonal antibodies to a target peptide); *Sutcliffe et al.*, *Science* (1983) 219:660-666 at 661-662 (teaching that “by following simple rules, one can in general select peptides that will elicit antibodies reactive with intact proteins”) (attached as Exhibit 23).

Appl. No. : 10/063,514  
Filed : May 1, 2002

In conclusion, the PTO's rejection based on lack of utility has been addressed above, and the PTO has otherwise failed to meet its burden to establish a reasonable basis to question the enablement provided for the claimed invention – unsupported conclusory statements are simply not sufficient. Given the skill in the art and the disclosure of how to make and use the claimed polypeptides, Applicants request that the PTO reconsider and withdraw its rejection under 35 U.S.C. § 112, first paragraph.

**Rejection under 35 U.S.C. §112, first paragraph – Written Description**

The PTO maintains the rejection of pending Claims 12-17 under 35 U.S.C. § 112, first paragraph, as failing to satisfy the written description requirement for the reasons set forth in the previous Office Actions. *Office Action* at 23.

**The PTO has Failed to Meet Its Initial Burden of Rebutting the Presumption that the Pending Claims are Adequately Described**

To overcome the presumption that the claimed subject matter is adequately described, the PTO must present “evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *Wertheim*, 541 F.2d at 263, 191 U.S.P.Q. at 97.” *M.P.E.P.* § 2163.04. To support its rejection of pending Claims 12-17, the PTO has merely repeated, nearly verbatim, the same arguments made in support of its enablement rejection.

Applicants note that Claims 4 and 5 which recited the limitations at issue have been canceled, and Claim 12, which depended from Claim 4, has been amended to depend from Claim 6. Claims 12-13 as dependent from Claim 6 do not recite percent amino acid sequence identity as a limitation, nor do they recite any limitation regarding differential expression in lung tumors. These claims are directed to fusion peptides of the disclosed sequence. In the absence of any arguments as to “why one of skill in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims,” the PTO has failed to rebut the presumption that the specification satisfies the written description requirement for pending Claims 12-13. *See M.P.E.P.* § 2163.04.



Appl. No. : 10/063,514  
Filed : May 1, 2002

With respect to Claims 14-17, which recite the limitation “wherein said isolated polypeptide or a fragment thereof can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO: 10 in lung tissue samples,” the PTO repeats its assertion that “[t]hese claims encompass any and all antigenically cross-reactive polypeptides possessing the recited percent identity, regardless of their biological activity.” *Id.* at 24. The PTO continues, arguing:

Applicants have not described the biologic activity of the PRO874 polypeptide or any of its variants. It is entirely unclear why the disclosure of a single polypeptide, i.e., PRO874, which is ideally suited to the making of antibodies to itself, would describe any and all antigenically cross-reactive polypeptides possessing the recited percent identity and possessing unknown and undisclosed biological activities, when the specification does not describe any biological activity. *Id.* at 24.

The PTO concludes that as a result, the claimed subject matter is not adequately described. *Id.*

As noted above, “[a] description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption.” *M.P.E.P.* § 2163.04 (emphasis added). Therefore “[t]he examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant’s disclosure a description of the invention defined by the claims.” *Id.*

The PTO has not provided any reasoning or evidence as to how the absence of the disclosure of “biological activity” results in an inadequate description of the subject matter of Claims 14-17. The claimed subject matter relates to polypeptides that have at least 95% sequence identity to SEQ ID NO:10, and can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO:10 in lung tissue samples. Applicants fail to see how any “biological activity” of the claimed polypeptides, aside from being able to generate an antibody which can specifically detect the polypeptide of SEQ ID NO:10, is at all relevant to an adequate description of the claimed polypeptides which are not claimed on the basis of any “biological activity.” In the absence of any other arguments as to why one of skill in the art would not recognize a description of the claimed invention in Applicants’ disclosure, the PTO has failed to rebut the presumption that the specification satisfies the written description requirement for Claims 14-17. *See M.P.E.P.* § 2163.04.

Appl. No. : 10/063,514  
Filed : May 1, 2002

Rejected Claims 12-17 are Adequately Described

The adequacy of written description support is a factual issue and is to be determined on a case-by-case basis. *See e.g., Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 U.S.P.Q.2d at 1116 (Fed. Cir. 1991). The factual determination in a written description analysis depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure. *Union Oil v. Atlantic Richfield Co.*, 208 F.3d 989, 996 (Fed. Cir. 2000).

Pending Claims 12-13, which depend from Claim 6, are adequately described by the specification. Claim 6 is directed to an isolated polypeptide comprising the specified portions of the amino acid sequence of the polypeptide of SEQ ID NO:10, or the amino acid sequence of the polypeptide encoded by the specified portion of the coding sequence of the cDNA deposited under ATCC accession number 209922.

As stated above, the PTO provides no basis for rejecting either of pending Claims 12-13 because the PTO's arguments are directed at claims reciting the limitation "wherein said isolated polypeptide is more highly expressed in normal lung tissue compared to lung tumor tissue..." or "wherein said isolated polypeptide or a fragment thereof can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO:10 in lung tissue samples." *See Office Action* at 23-24. As amended, Claims 12-13 depend from Claim 6, which does not recite the objected to limitation.

Applicants assert that each recited element of Claim 6 is explicitly disclosed in the specification, either in writing (*see, e.g., Specification* at Figure 10) or by virtue of a biological deposit. Accordingly, there can be no basis for holding that Claim 6 is not adequately described. Likewise, Claims 12-13, which are drawn to chimeric polypeptides comprising the polypeptide of Claim 6, are also fully described by the specification. As such, Applicants request that the PTO reconsider and withdraw the rejection of Claims 12-13 under 35 U.S.C. § 112, first paragraph, for lack of written description.

Claims 14-17 are also adequately described by the specification. Claim 14 is directed to an isolated polypeptide having at least 95% amino acid sequence identity to the recited amino acid sequence of the polypeptide of SEQ ID NO:10, or the amino acid sequence of the polypeptide encoded by the recited coding sequence of the cDNA deposited under ATCC accession number 209922; wherein said isolated polypeptide or a fragment thereof can be used to

Appl. No. : 10/063,514  
Filed : May 1, 2002

generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO: 10 in lung tissue samples. Claims 16 and 17 ultimately depend from Claim 14. Similarly, Claim 15 recites "at least 99% amino acid sequence identity."

Applicants maintain that there is no substantial variation within the species which fall within the scope of the rejected claims, which require at least 95% amino acid sequence identity to SEQ ID NO:10 and can be used to generate antibodies which specifically detect the polypeptide of SEQ ID NO:10 in lung tissue samples. As such, Applicants were in possession of the common attributes or features of the claimed subject matter.

The rejected claims are analogous to the claims discussed in Example 14 of the written description training materials available on the PTO's website. In Example 14, the written description requirement was found to be satisfied for claims directed to polypeptides with 95% homology to a disclosed sequence that also possess a recited catalytic activity, where procedures for making variant proteins were routine in the art and the specification provided an assay for detecting the recited catalytic activity of the protein. This disclosure satisfies the written description requirement even though the applicant had disclosed only a single species and had not made any variants. The Guidelines state that "[t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity."

Similarly, the pending claims also have at least 95% or 99% sequence identity to the disclosed sequence, and must be able to generate antibodies which specifically detect the polypeptide of SEQ ID NO:10 in lung tissue samples. As in Example 14, at the time of the effective filing date of the instant application, it was well known in the art how to make polypeptides with at least 95% or 99% amino acid sequence identity to the disclosed sequences. *See, e.g., Specification* at ¶¶ [0256]-[0271]. In addition, the specification discloses in detail how to make antibodies which specifically detect a particular PRO polypeptide, and how to use them to detect the PRO polypeptide in a particular tissue. *See, e.g., Specification* ¶¶ [0363]-[0379], [0407], and [0493]-[0499]. Like a particular catalytic activity, the function of being useful to produce an antibody specific to SEQ ID NO:10 is directly related to the structure of the claimed

**Appl. No.** : 10/063,514  
**Filed** : May 1, 2002

polypeptides. Thus, like Example 14, the genus of polypeptides that have at least 95% amino acid sequence identity to the disclosed sequences and possess the described functional activity are adequately described.

Claims 16 and 17, drawn to particular embodiments of Claim 14, are also fully described by the specification. The PTO does not contest the written description support for any embodiment recited in Claims 16-17.

The PTO asserts that the present claims are not analogous to those discussed in Example 14 of the written description guidelines because the specification does not describe any biological activity of the claimed polypeptides and because the claims are not limited to any specific "biological activity" of the claimed polypeptides. *Office Action* at 24-25.

Applicants submit that the applicability of Example 14 is not limited to polypeptides for which the biological function is known and recited, but extends to all situations where the polypeptide is useful and there is no substantial variation within the species encompassed by the claims. The purpose of the recited catalytic activity in the example is to limit the amount of structural variation within the species. The commentary in the Guidelines states that the description of an assay to detect variants which have the recited activity, along with 95% homology, is sufficient to satisfy the written description requirement.

Similarly, in the instant case, Claims 14-17 must share a particular "biological activity" which restricts the amount of permissible structural variation within the species – the claimed polypeptides must be useable to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO:10 in lung tissue samples. This limitation combined with the disclosure of how to make and test the recited antibodies generated from the claimed polypeptides, along with the requirement of least 95% or 99% amino acid sequence identity, results in claimed subject matter where there is no substantial variation within the species encompassed by the claims. Accordingly, Applicants maintain that the pending claims are analogous to the claims in Example 14.

As for the PTO's statement that "[i]t is entirely unclear why the disclosure of a single polypeptide, i.e., PRO874, ...would describe any and all antigenically cross-reactive polypeptides possessing the recited percent identity," the basic premise that a large genus can not

Appl. No. : 10/063,514  
Filed : May 1, 2002

be adequately described by a single species is simply wrong. In a recent Federal Circuit decision, *In re Wallach*, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004), the Court stated:

[W]e agree with Appellants that the state of the art has developed such that the complete amino acid sequence of a protein may put one in possession of the genus of DNA sequences encoding it, and that one of ordinary skill in the art at the time the '129 application was filed may have therefore been in possession of the entire genus of DNA sequences that can encode the disclosed partial protein sequence, even if individual species within that genus might not have been described or rendered obvious. ... A claim to the genus of DNA molecules complementary to the RNA having the sequences encompassed by that formula, even if defined only in terms of the protein sequence that the DNA molecules encode, while containing a large number of species, is definite in scope and provides the public notice required of patent applicants.

\*\*\*

Moreover, we see no reason to require a patent applicant to list every possible permutation of the nucleic acid sequences that can encode a particular protein for which the amino acid sequence is disclosed, given the fact that it is, as explained above, a routine matter to convert back and forth between an amino acid sequence and the sequences of the nucleic acid molecules that can encode it. *Id.* (emphasis added).

The Court did not require the applicants in *Wallach* to actually make or individually describe all of the *vast* number of sequences which encode the disclosed protein sequence. This is in spite of the fact that only a single protein sequence was disclosed, and the encompassed genus was enormous due to codon degeneracy in the genetic code – even the most skilled artisan could not individually envision the detailed chemical structure of the nucleic acids encompassed by the claimed genus. The Court reasoned that because it is routine to convert between amino acid sequences and nucleic acid sequences, disclosure of a single amino acid sequence was sufficient to place the applicants in possession of the enormous genus of nucleic acids which could encode the sequence.

The facts in *Wallach* are very similar to the instant case. Here, Applicants have disclosed SEQ ID NO:10, and claim polypeptides which are at least 95% or 99% identical to it and have the functional limitation of the ability to generate antibodies which can be used to specifically detect SEQ ID NO:10 in lung tissue samples. As discussed above, it is routine in the art to create polypeptides which have at least 95% or 99% sequence identity to SEQ ID NO:10 – it is just as

Appl. No. : 10/063,514  
Filed : May 1, 2002

predictable and easy as creating all of the nucleic acids which encode a particular amino acid sequence. Similarly, it is well within the knowledge of those skilled in the art how to determine which polypeptides can be used to make the recited antibodies. The predictability of this structure/function combination is sufficient to place the claimed subject matter in the possession of the Applicants, and thus the claimed polypeptides are adequately described. The *Wallach* opinion makes clear that there is no need to literally describe more than a single species to adequately describe a large genus where one of skill in the art recognizes that the disclosed species puts the applicant in possession of the claimed genus.

In conclusion, the PTO has failed to meet its “initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant’s disclosure a description of the invention defined by the claims.” *M.P.E.P.* § 2163.04. And even if it has met this burden, Applicants submit that they have satisfied the written description requirement for the pending claims based on the actual reduction to practice of SEQ ID NO:10, by specifying a high level of amino acid sequence identity, and by describing how to make antibodies to the disclosed sequence, all of which result in a lack of substantial variability in the species falling within the scope of the instant claims. Applicants submit that this disclosure would allow one of skill in the art to “recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus.” Hence, Applicants respectfully request that the PTO reconsider and withdraw the written description rejection under 35 U.S.C. §112.

**Rejections under 35 U.S.C. § 112, first paragraph – Written Description, New Matter**

The PTO has rejected pending Claims 6-7, 9 and 11-17 under 35 U.S.C. §112, first paragraph, as containing new matter. *Office Action* at 25-27. Regarding amino acid species starting at methionine residue #34, the PTO argues that there is no “express, implicit, or inherent support for this species to the exclusion of all other species.” *Id.* at 25. Regarding the limitations reciting residues 81-109 and 232-253 of SEQ ID NO:10, the PTO argues that “the specification does not disclose a fragment of a PRO polypeptide that is the intracellular domain. The newly added limitations ‘81-109’ and ‘232-253’ imply that ‘81-109’ and ‘232-253’ are the extracellular domains.” *Id.* at 26-27.

Appl. No. : 10/063,514  
Filed : May 1, 2002

The PTO's Arguments Fail to Overcome the Presumption that the Claimed Invention is Adequately Described in the Specification

Paragraph [0196] of the specification states that "it is conceivable and possible that other methionine residues located either upstream or downstream from the amino acid position 1 in the figures may be employed as the starting amino acid residue for the PRO polypeptides." *Specification* at ¶ [0196] (emphasis added). Figure 10 and SEQ ID NO:10 show 8 methionine residues in the sequence. Combining the statement in paragraph [0196] with the disclosure of SEQ ID NO:10, Applicants have conveyed with reasonable clarity to those skilled in the art that, as of the filing date sought, Applicants were in possession of polypeptides of SEQ ID NO:10 beginning at any of the methionine residues listed in SEQ ID NO:10. One of these polypeptides is the one which begins with the methionine at residue #34. Therefore, Applicants were clearly in possession of "the polypeptide having the amino acid sequence of amino acids 34-321 of SEQ ID NO: 10" at the time of filing.

Applicants have also described in Figure 9, SEQ ID NO:9 and the cDNA deposited under ATCC accession number 209922, a cDNA sequence which encodes the entirety of the polypeptide of SEQ ID NO:10. As such, SEQ ID NO:9 inherently discloses the coding sequence of the polypeptides of SEQ ID NO:10 which start at any of the eight methionine residues, including the polypeptide which begins at methionine #34. One of skill in the art would clearly recognize that methionine #34 is encoded by the codon beginning at nucleotide 100 of SEQ ID NO:9, and that the stop codon ends at nucleotide 966. Therefore, the polypeptide of SEQ ID NO:10 which begins at residue #34 is encoded by nucleotides 100-966 of SEQ ID NO:9 and cDNA deposited under ATCC accession number 209922. Applicants were therefore clearly in possession of "the amino acid sequence of the polypeptide encoded by nucleotides 100-966 of the cDNA deposited under ATCC accession number 209922" at the time of filing.

The PTO acknowledges that paragraph [0196] discloses that methionine residues upstream or downstream of the amino acid in position 1 may be the start amino acid. However, the PTO appears to argue that because there is more than one methionine residue in SEQ ID NO:10, Applicants have not adequately described any of the possible polypeptides of SEQ ID NO:10 beginning at a methionine residue. The PTO states that "the species methionine residue

Appl. No. : 10/063,514  
Filed : May 1, 2002

#34 as the starting amino acid is not supported by this generic disclosure because there is no express, implicit, or inherent support for this species to the exclusion of all the other species. In other words, there is no evidence that the disclosure would reasonably lead the skilled artisan to this particular species.” *Office Action* at 25 (emphasis added.)

Applicants submit that this argument misstates the test for compliance with the written description requirement. The test is “whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.” *M.P.E.P.* §2163.02 (internal citations omitted, emphasis added). Clearly, as discussed above, at the time of filing Applicants were in possession of the polypeptide of SEQ ID NO:10 starting at methionine #34 and the nucleic acid sequence which encodes this polypeptide. Contrary to the PTO’s assertion, where Applicants have adequately described several polypeptides related to SEQ ID NO:10, there is nothing in the written description requirement which prevents the Applicants from claiming only one of them.

Therefore, Applicants submit that the PTO has failed to meet its initial burden of “presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims” with regard to this subject matter. *M.P.E.P.* §2163.04 (internal citations omitted, emphasis added).

Finally, Figure 10 discloses the following fragments of SEQ ID NO:10: amino acids 57-80, 110-126, 215-231, and 254-274. Implicit in the disclosure of fragments 57-80 and 110-126 of SEQ ID NO:10 is the disclosure of the fragment 81-109 of SEQ ID NO:10. Likewise, the disclosure of fragments 110-126 and 215-231 of SEQ ID NO:10 implicitly discloses the fragment 232-253 of SEQ ID NO:10. Therefore, in light of Figure 10, Applicants were clearly in possession of the subject matter “the polypeptide having the amino acid sequence of amino acids 81-109 or 232-253 of SEQ ID NO: 10” at the time of filing.

The PTO argues that while Figure 10 discloses that SEQ ID NO:10 possesses several transmembrane domains, the extracellular domains depend on how the polypeptide is arranged in the membrane, and that the amendments imply that “81-109” and “232-253” are the extracellular domains. The PTO concludes that “[s]upport for the one arrangement implied by the present limitations cannot be found in the disclosure as originally filed. Hence, the newly added limitations constitute new matter.” *Office Action* at 26-27.



Appl. No. : 10/063,514  
Filed : May 1, 2002

Applicants submit that this rejection is improper because it is a rejection not of the subject matter that is claimed, but rather what the PTO believes is “implied” by the claims. The disputed portion of the claims reads “An isolated polypeptide comprising:... (b) the amino acid sequence of the polypeptide having the amino acid sequence of amino acids 81-109 or 232-253 of SEQ ID NO: 10.” Nothing in this claim suggests or implies that the claimed portions of SEQ ID NO:10 are intracellular, extracellular or transmembrane portions of the protein. Applicants remind the PTO that the test for written description on which a new matter rejection is based is “whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.” *M.P.E.P.* §2163.02 (internal citations omitted, emphasis added). Thus, the PTO is improperly reading limitations into the claim that are not present, and its rejection is therefore improper since it is not directed to the claimed subject matter, but to what the PTO feels is “implied” in the claims. In the absence of any other arguments as to why one of skill in the art would not recognize a description of the claimed invention in Applicants’ disclosure, the PTO has failed to rebut the presumption that the specification satisfies the written description requirement with respect to this subject matter. *See M.P.E.P.* § 2163.04.

In conclusion, the Applicants respectfully submit that the PTO has failed to rebut the presumption that the amendments to the claims are adequately described, as it has failed to provide any evidence or reasoning to support the rejection: “[a] description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the PTO to rebut the presumption.” *M.P.E.P.* § 2163.04 (emphasis added). Applicants submit that the instant disclosure “conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.” Hence, Applicants respectfully request that the PTO reconsider and withdraw the new matter rejection of Claims 6-7, 9 and 11-17 under 35 U.S.C. §112, first paragraph.

Appl. No. : 10/063,514  
Filed : May 1, 2002

### CONCLUSION

In view of the above, Applicants respectfully maintain that claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

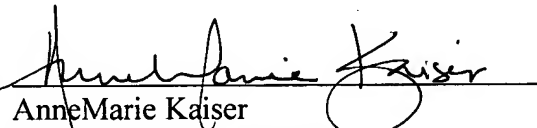
Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated:

July 14, 2006

By:

  
AnneMarie Kaiser  
Registration No. 37,649  
Attorney of Record  
Customer No. 30,313  
(619) 235-8550

2673090  
061206

## EXHIBIT 1

Proteomics. 2005 Mar;5(4):894-906.

[Related Articles, Links](#)



### **A transcriptomic and proteomic analysis of the effect of CpG-ODN on human THP-1 monocytic leukemia cells.**

**Kuo CC, Kuo CW, Liang CM, Liang SM.**

Institute of BioAgricultural Sciences, Academia Sinica, Taipei, Taiwan.

The CpG motif of bacterial DNA (CpG-DNA) is a potent immunostimulating agent whose mechanism of action is not yet clear. Here, we used both DNA microarray and proteomic approaches to investigate the effects of oligodeoxynucleotides containing the CpG motif (CpG-ODN) on gene transcription and protein expression profiles of CpG-ODN responsive THP-1 cells. Microarray analysis revealed that 2 h stimulation with CpG-ODN up-regulated 50 genes and down-regulated five genes. These genes were identified as being associated with inflammation, antimicrobial defense, transcriptional regulation, signal transduction, tumor progression, cell differentiation, proteolysis and metabolism. Longer stimulation (8 h) with CpG-ODN enhanced transcriptional expression of 58 genes. Among these 58 genes, none except one, namely WNT1 inducible signaling pathway protein 2, was the same as those induced after 2 h stimulation. Proteomic analysis by two-dimensional gel electrophoresis, followed by mass spectrometry identified several proteins up-regulated by CpG-ODN. These proteins included heat shock proteins, modulators of inflammation, metabolic proteins and energy pathway proteins. Comparison of microarray and proteomic expression profiles showed poor correlation. Use of more reliable and sensitive analyses, such as reverse transcriptase polymerase chain reaction, Western blotting and functional assays, on several genes and proteins, nonetheless, confirmed that there is indeed good correlation between mRNA and protein expression after CpG-ODN treatment. This study also revealed that several anti-apoptotic and neuroprotective related proteins, not previously reported, are activated by CpG-DNA. These findings have extended our knowledge on the activation of cells by CpG-DNA and may contribute to further understanding of mechanisms that link innate immunity with acquired immune response(s).

PMID: 15693060 [PubMed - indexed for MEDLINE]

## EXHIBIT 2

### SECOND DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I am currently employed by Genentech, Inc. where my job title is Staff Scientist.
2. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.
3. As I stated in my previous Declaration dated May 7, 2004 (attached as Exhibit A), my laboratory has been employing a variety of techniques, including microarray analysis, to identify genes which are differentially expressed in human tumor tissue relative to normal human tissue. The primary purpose of this research is to identify proteins that are abundantly expressed on certain human tumor tissue(s) and that are either (i) not expressed, or (ii) expressed at detectably lower levels, on normal tissue(s).
4. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor tissue at significantly higher levels than in normal human tissue. To date, we have successfully generated antibodies that bind to 31 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human tumor tissue and normal tissue. We have then quantitatively compared the levels of mRNA and protein in both the tumor and normal tissues analyzed. The results of these analyses are attached herewith as Exhibit B. In Exhibit B, "+" means that the mRNA or protein was detectably overexpressed in the tumor tissue relative to normal tissue and "-" means that no detectable overexpression was observed in the tumor tissue relative to normal tissue.
5. As shown in Exhibit B, of the 31 genes identified as being detectably overexpressed in human tumor tissue as compared to normal human tissue at the mRNA level, 28 of them (i.e., greater than 90%) are also detectably overexpressed in human tumor tissue as compared to normal human tissue at the protein level. As such, in the cases where we have been able to quantitatively measure both (i) mRNA and (ii) protein levels in both (i) tumor tissue and (ii) normal tissue, we have observed that in the vast majority of cases, there is a very strong correlation between increases in mRNA expression and increases in the level of protein encoded by that mRNA.

6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4-5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor tissue relative to a normal tissue more often than not correlates to a similar increase in abundance of the encoded protein in the tumor tissue relative to the normal tissue. In fact, it remains a generally accepted working assumption in molecular biology that increased mRNA levels are more often than not predictive of elevated levels of the encoded protein. In fact, an entire industry focusing on the research and development of therapeutic antibodies to treat a variety of human diseases, such as cancer, operates on this working assumption.
7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 3-29-00

By: Paul Polakis  
Paul Polakis, Ph.D.

EXHIBIT A

DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I was awarded a Ph.D. by the Department of Biochemistry of the Michigan State University in 1984. My scientific Curriculum Vitae is attached to and forms part of this Declaration (Exhibit A).
2. I am currently employed by Genentech, Inc. where my job title is Staff Scientist. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.
3. As part of the Tumor Antigen Project, my laboratory has been analyzing differential expression of various genes in tumor cells relative to normal cells. The purpose of this research is to identify proteins that are abundantly expressed on certain tumor cells and that are either (i) not expressed, or (ii) expressed at lower levels, on corresponding normal cells. We call such differentially expressed proteins "tumor antigen proteins". When such a tumor antigen protein is identified, one can produce an antibody that recognizes and binds to that protein. Such an antibody finds use in the diagnosis of human cancer and may ultimately serve as an effective therapeutic in the treatment of human cancer.
4. In the course of the research conducted by Genentech's Tumor Antigen Project, we have employed a variety of scientific techniques for detecting and studying differential gene expression in human tumor cells relative to normal cells, at genomic DNA, mRNA and protein levels. An important example of one such technique is the well known and widely used technique of microarray analysis which has proven to be extremely useful for the identification of mRNA molecules that are differentially expressed in one tissue or cell type relative to another. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor cells at significantly higher levels than in corresponding normal human cells. To date, we have generated antibodies that bind to about 30 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human cancer cells and corresponding normal cells. We have then compared the levels of mRNA and protein in both the tumor and normal cells analyzed.
5. From the mRNA and protein expression analyses described in paragraph 4 above, we have observed that there is a strong correlation between changes in the level of mRNA present in any particular cell type and the level of protein

expressed from that mRNA in that cell type. In approximately 80% of our observations we have found that increases in the level of a particular mRNA correlates with changes in the level of protein expressed from that mRNA when human tumor cells are compared with their corresponding normal cells.

6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4 and 5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor cell relative to a normal cell typically correlates to a similar increase in abundance of the encoded protein in the tumor cell relative to the normal cell. In fact, it remains a central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded protein. While there have been published reports of genes for which such a correlation does not exist, it is my opinion that such reports are exceptions to the commonly understood general rule that increased mRNA levels are predictive of corresponding increased levels of the encoded protein.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 5/07/04

By: Paul Polakis  
Paul Polakis, Ph.D.

## CURRICULUM VITAE

PAUL G. POLAKIS  
Staff Scientist  
Genentech, Inc  
1 DNA Way, MS#40  
S. San Francisco, CA 94080

### EDUCATION:

Ph.D., Biochemistry, Department of Biochemistry,  
Michigan State University (1984)

B.S., Biology. College of Natural Science, Michigan State University (1977)

### PROFESSIONAL EXPERIENCE:

2002-present	Staff Scientist, Genentech, Inc S. San Francisco, CA
1999- 2002	Senior Scientist, Genentech, Inc., S. San Francisco, CA
1997 -1999	Research Director Onyx Pharmaceuticals, Richmond, CA
1992- 1996	Senior Scientist, Project Leader, Onyx Pharmaceuticals, Richmond, CA
1991-1992	Senior Scientist, Chiron Corporation, Emeryville, CA.
1989-1991	Scientist, Cetus Corporation, Emeryville CA.
1987-1989	Postdoctoral Research Associate, Genentech, Inc., South San Francisco, CA.
1985-1987	Postdoctoral Research Associate, Department of Medicine, Duke University Medical Center, Durham, NC



1984-1985

Assistant Professor, Department of Chemistry,  
Oberlin College, Oberlin, Ohio

1980-1984

Graduate Research Assistant, Department of  
Biochemistry, Michigan State University  
East Lansing, Michigan

**PUBLICATIONS:**

1. Polakis, P. G. and Wilson, J. E. 1982 Purification of a Highly Bindable Rat Brain Hexokinase by High Performance Liquid Chromatography. **Biochem. Biophys. Res. Commun.** 107, 937-943.

2. Polakis, P.G. and Wilson, J. E. 1984 Proteolytic Dissection of Rat Brain Hexokinase: Determination of the Cleavage Pattern during Limited Digestion with Trypsin. **Arch. Biochem. Biophys.** 234, 341-352.

3. Polakis, P. G. and Wilson, J. E. 1985 An Intact Hydrophobic N-Terminal Sequence is Required for the Binding Rat Brain Hexokinase to Mitochondria. **Arch. Biochem. Biophys.** 236, 328-337.

4. Uhing, R.J., Polakis, P.G. and Snyderman, R. 1987 Isolation of GTP-binding Proteins from Myeloid HL60 Cells. **J. Biol. Chem.** 262, 15575-15579.

5. Polakis, P.G., Uhing, R.J. and Snyderman, R. 1988 The Formylpeptide Chemoattractant Receptor Copurifies with a GTP-binding Protein Containing a Distinct 40 kDa Pertussis Toxin Substrate. **J. Biol. Chem.** 263, 4969-4979.

6. Uhing, R. J., Dillon, S., Polakis, P. G., Truett, A. P. and Snyderman, R. 1988 Chemoattractant Receptors and Signal Transduction Processes in Cellular and Molecular Aspects of Inflammation ( Poste, G. and Crooke, S. T. eds.) pp 335-379.

7. Polakis, P.G., Evans, T. and Snyderman 1989 Multiple Chromatographic Forms of the Formylpeptide Chemoattractant Receptor and their Relationship to GTP-binding Proteins. **Biochem. Biophys. Res. Commun.** 161, 276-283.

8. Polakis, P. G., Snyderman, R. and Evans, T. 1989 Characterization of G25K, a GTP-binding Protein Containing a Novel Putative Nucleotide Binding Domain. **Biochem. Biophys. Res. Commun.** 160, 25-32.

9. Polakis, P., Weber, R.F., Nevins, B., Didsbury, J. Evans, T. and Snyderman, R. 1989 Identification of the *ral* and *rac1* Gene Products, Low Molecular Mass GTP-binding Proteins from Human Platelets. **J. Biol. Chem.** 264, 16383-16389.

10. Snyderman, R., Perianin, A., Evans, T., Polakis, P. and Didsbury, J. 1989 G Proteins and Neutrophil Function. In ADP-Ribosylating Toxins and G Proteins: Insights into Signal Transduction. ( J. Moss and M. Vaughn, eds.) Amer. Soc. Microbiol. pp. 295-323.

11. Hart, M.J., Polakis, P.G., Evans, T. and Cerrione, R.A. 1990 The Identification and Characterization of an Epidermal Growth Factor-Stimulated Phosphorylation of a Specific Low Molecular Mass GTP-binding Protein in a Reconstituted Phospholipid Vesicle System. *J. Biol. Chem.* 265, 5990-6001.
12. Yatani, A., Okabe, K., Polakis, P., Halenbeck, R., McCormick, F. and Brown, A. M. 1990 ras p21 and GAP Inhibit Coupling of Muscarinic Receptors to Atrial K<sup>+</sup> Channels. *Cell* 61, 769-776.
13. Munemitsu, S., Innis, M.A., Clark, R., McCormick, F., Ullrich, A. and Polakis, P.G. 1990 Molecular Cloning and Expression of a G25K cDNA, the Human Homolog of the Yeast Cell Cycle Gene CDC42. *Mol. Cell. Biol.* 10, 5977-5982.
14. Polakis, P.G., Rubinfeld, B., Evans, T. and McCormick, F. 1991 Purification of Plasma Membrane-Associated GTPase Activating Protein (GAP) Specific for rap-1/krev-1 from HL60 Cells. *Proc. Natl. Acad. Sci. USA* 88, 239-243.
15. Moran, M. F., Polakis, P., McCormick, F., Pawson, T. and Ellis, C. 1991 Protein Tyrosine Kinases Regulate the Phosphorylation, Protein Interactions, Subcellular Distribution, and Activity of p21ras GTPase Activating Protein. *Mol. Cell. Biol.* 11, 1804-1812.
16. Rubinfeld, B., Wong, G., Bekesi, E., Wood, A., McCormick, F. and Polakis, P. G. 1991 A Synthetic Peptide Corresponding to a Sequence in the GTPase Activating Protein Inhibits p21<sup>ras</sup> Stimulation and Promotes Guanine Nucleotide Exchange. *Internatl. J. Peptide and Prot. Res.* 38, 47-53.
17. Rubinfeld, B., Munemitsu, S., Clark, R., Conroy, L., Watt, K., Crosier, W., McCormick, F., and Polakis, P. 1991 Molecular Cloning of a GTPase Activating Protein Specific for the Krev-1 Protein p21<sup>rap1</sup>. *Cell* 65, 1033-1042.
18. Zhang, K., Papageorge, A., G., Martin, P., Vass, W. C., Olah, Z., Polakis, P., McCormick, F. and Lowy, D. R. 1991 Heterogenous Amino Acids in RAS and Rap1A Specifying Sensitivity to GAP Proteins. *Science* 254, 1630-1634.
19. Martin, G., Yatani, A., Clark, R., Polakis, P., Brown, A. M. and McCormick, F. 1992 GAP Domains Responsible for p21<sup>ras</sup>-dependent Inhibition of Muscarinic Atrial K<sup>+</sup> Channel Currents. *Science* 255, 192-194.
20. McCormick, F., Martin, G. A., Clark, R., Bollag, G. and Polakis, P. 1992 Regulation of p21ras by GTPase Activating Proteins. *Cold Spring Harbor Symposia on Quantitative Biology*. Vol. 56, 237-241.
21. Pronk, G. B., Polakis, P., Wong, G., deVries-Smits, A. M., Bos J. L. and McCormick, F. 1992 p60<sup>v-src</sup> Can Associate with and Phosphorylate the p21<sup>ras</sup> GTPase Activating Protein. *Oncogene* 7, 389-394.
22. Polakis P. and McCormick, F. 1992 Interactions Between p21<sup>ras</sup> Proteins and Their GTPase Activating Proteins. In Cancer Surveys ( Franks, L. M., ed.) 12, 25-42.

23. Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M., Polakis, P. and McCormick, F. 1992 Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. *Cell* 69, 551-558.
24. Polakis, P., Rubinfeld, B. and McCormick, F. 1992 Phosphorylation of rap1GAP in vivo and by cAMP-dependent Kinase and the Cell Cycle p34<sup>cdc2</sup> Kinase in vitro. *J. Biol. Chem.* 267, 10780-10785.
25. McCabe, P.C., Haubrauck, H., Polakis, P., McCormick, F., and Innis, M. A. 1992 Functional Interactions Between p21<sup>rap1A</sup> and Components of the Budding pathway of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 12, 4084-4092.
26. Rubinfeld, B., Crosier, W.J., Albert, I., Conroy, L., Clark, R., McCormick, F. and Polakis, P. 1992 Localization of the rap1GAP Catalytic Domain and Sites of Phosphorylation by Mutational Analysis. *Mol. Cell. Biol.* 12, 4634-4642.
27. Ando, S., Kaibuchi, K., Sasaki, K., Hiraoka, T., Nishiyama, T., Mizuno, T., Asada, M., Nunoi, H., Matsuda, I., Matsuura, Y., Polakis, P., McCormick, F. and Takai, Y. 1992 Post-translational processing of rac p21s is important both for their interaction with the GDP/GTP exchange proteins and for their activation of NADPH oxidase. *J. Biol. Chem.* 267, 25709-25713.
28. Janoueix-Lerosey, I., Polakis, P., Tavitian, A. and deGunzberg, J. 1992 Regulation of the GTPase activity of the ras-related rap2 protein. *Biochem. Biophys. Res. Commun.* 189, 455-464.
29. Polakis, P. 1993 GAPs Specific for the rap1/Krev-1 Protein. in GTP-binding Proteins: the ras-superfamily. (J.C. LaCale and F. McCormick, eds.) 445-452.
30. Polakis, P. and McCormick, F. 1993 Structural requirements for the interaction of p21<sup>ras</sup> with GAP, exchange factors, and its biological effector target. *J. Biol. Chem.* 268, 9157-9160.
31. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S., Masiarz, F., Munemitsu, S. and Polakis, P. 1993 Association of the APC gene product with beta-catenin. *Science* 262, 1731-1734.
32. Weiss, J., Rubinfeld, B., Polakis, P., McCormick, F., Cavenhee, W. A. and Arden, K. 1993 The gene for human rap1-GTPase activating protein (rap1GAP) maps to chromosome 1p35-1p36.1. *Cytogenet. Cell Genet.* 66, 18-21.
33. Sato, K. Y., Polakis, P., Haubruck, H., Fasching, C. L., McCormick, F. and Stanbridge, E. J. 1994 Analysis of the tumor suppressor activity of the K-ras gene in human tumor cell lines. *Cancer Res.* 54, 552-559.
34. Janoueix-Lerosey, I., Fontenay, M., Tobelem, G., Tavitian, A., Polakis, P. and DeGunzburg, J. 1994 Phosphorylation of rap1GAP during the cell cycle. *Biochem. Biophys. Res. Commun.* 202, 967-975.
35. Munemitsu, S., Souza, B., Mueller, O., Albert, I., Rubinfeld, B., and Polakis, P. 1994 The APC gene product associates with microtubules in vivo and affects their assembly in vitro. *Cancer Res.* 54, 3676-3681.

36. Rubinfeld, B. and Polakis, P. 1995 Purification of baculovirus produced rap1GAP. *Methods Enz.* 255,31
37. Polakis, P. 1995 Mutations in the APC gene and their implications for protein structure and function. *Current Opinions in Genetics and Development* 5, 66-71
38. Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S. and Polakis P. 1995 The APC protein and E-cadherin form similar but independent complexes with  $\alpha$ -catenin,  $\beta$ -catenin and Plakoglobin. *J. Biol. Chem.* 270, 5549-5555
39. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. 1995 Regulation of intracellular  $\beta$ -catenin levels by the APC tumor suppressor gene. *Proc. Natl. Acad. Sci.* 92, 3046-3050.
40. Lock, P., Fumagalli, S., Polakis, P. McCormick, F. and Courtneidge, S. A. 1996 The human p62 cDNA encodes Sam68 and not the rasGAP-associated p62 protein. *Cell* 84, 23-24.
41. Papkoff, J., Rubinfeld, B., Schryver, B. and Polakis, P. 1996 Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell. Biol.* 16, 2128-2134.
42. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. 1996 Binding of GSK3 $\beta$  to the APC- $\beta$ -catenin complex and regulation of complex assembly. *Science* 272, 1023-1026.
43. Munemitsu, S., Albert, I., Rubinfeld, B. and Polakis, P. 1996 Deletion of amino-terminal structure stabilizes  $\beta$ -catenin in vivo and promotes the hyperphosphorylation of the APC tumor suppressor protein. *Mol. Cell. Biol.* 16, 4088-4094.
44. Hart, M. J., Callow, M. G., Sousa, B. and Polakis P. 1996 IQGAP1, a calmodulin binding protein with a rasGAP related domain, is a potential effector for cdc42Hs. *EMBO J.* 15, 2997-3005.
45. Nathke, I. S., Adams, C. L., Polakis, P., Sellin, J. and Nelson, W. J. 1996 The adenomatous polyposis coli (APC) tumor suppressor protein is localized to plasma membrane sites involved in active epithelial cell migration. *J. Cell. Biol.* 134, 165-180.
46. Hart, M. J., Sharma, S., elMasry, N., Qui, R-G., McCabe, P., Polakis, P. and Bollag, G. 1996 Identification of a novel guanine nucleotide exchange factor for the rho GTPase. *J. Biol. Chem.* 271, 25452.
47. Thomas JE, Smith M, Rubinfeld B, Gutowski M, Beckmann RP, and Polakis P. 1996 Subcellular localization and analysis of apparent 180-kDa and 220-kDa proteins of the breast cancer susceptibility gene, BRCA1. *J. Biol. Chem.* 1996 271, 28630-28635
48. Hayashi, S., Rubinfeld, B., Souza, B., Polakis, P., Wieschaus, E., and Levine, A. 1997 A Drosophila homolog of the tumor suppressor adenomatous polyposis coli

down-regulates  $\beta$ -catenin but its zygotic expression is not essential for the regulation of armadillo. *Proc. Natl. Acad. Sci.* 94, 242-247.

49. Vlemingcx, K., Rubinfeld, B., Polakis, P. and Gumbiner, B. 1997 The APC tumor suppressor protein induces a new axis in *Xenopus* embryos. *J. Cell. Biol.* 136, 411-420.

50. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, P. and Polakis, P. 1997 Stabilization of  $\beta$ -catenin by genetic defects in melanoma cell lines. *Science* 275, 1790-1792.

51. Polakis, P. The adenomatous polyposis coli (APC) tumor suppressor. 1997. *Biochem. Biophys. Acta*, 1332, F127-F147.

52. Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S., and Polakis, P. 1997 Loss of  $\beta$ -catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. *Cancer Res.* 57, 4624-4630.

53. Porfiri, E., Rubinfeld, B., Albert, I., Hovanes, K., Waterman, M., and Polakis, P. 1997 Induction of a  $\beta$ -catenin-LEF-1 complex by wnt-1 and transforming mutants of  $\beta$ -catenin. *Oncogene* 15, 2833-2839.

54. Thomas JE, Smith M, Tonkinson JL, Rubinfeld B, and Polakis P., 1997 Induction of phosphorylation on BRCA1 during the cell cycle and after DNA damage. *Cell Growth Differ.* 8, 801-809.

55. Hart, M., de los Santos, R., Albert, I., Rubinfeld, B., and Polakis P., 1998 Down regulation of  $\beta$ -catenin by human Axin and its association with the adenomatous polyposis coli (APC) tumor suppressor,  $\beta$ -catenin and glycogen synthase kinase 3 $\beta$ . *Current Biology* 8, 573-581.

56. Polakis, P. 1998 The oncogenic activation of  $\beta$ -catenin. *Current Opinions in Genetics and Development* 9, 15-21

57. Matt Hart, Jean-Paul Concordet, Irina Lassot, Iris Albert, Rico del los Santos, Herve Durand, Christine Perret, Bonnee Rubinfeld, Florence Margottin, Richard Benarous and Paul Polakis. 1999 The F-box protein  $\beta$ -TrCP associates with phosphorylated  $\beta$ -catenin and regulates its activity in the cell. *Current Biology* 9, 207-10.

58. Howard C. Crawford, Barbara M. Fingleton, Bonnee Rubinfeld, Paul Polakis and Lynn M. Matrisian. 1999 The metalloproteinase matrilysin is a target of  $\beta$ -catenin transactivation in intestinal tumours. *Oncogene* 18, 2883-91.

59. Meng J, Glick JL, Polakis P, Casey PJ. 1999 Functional interaction between Galpha(z) and Rap1GAP suggests a novel form of cellular cross-talk. *J Biol Chem.* 17, 36663-9

60. Vijayasurian Easwaran, Virginia Song, Paul Polakis and Steve Byers 1999 The ubiquitin-proteosome pathway and serine kinase activity modulate APC mediated regulation of  $\beta$ -catenin-LEF signaling. *J. Biol. Chem.* 274(23):16641-5.
61. Polakis P, Hart M and Rubinfeld B. 1999 Defects in the regulation of beta-catenin in colorectal cancer. *Adv Exp Med Biol.* 470, 23-32
62. Shen Z, Batzer A, Koehler JA, Polakis P, Schlessinger J, Lydon NB, Moran MF. 1999 Evidence for SH3 domain directed binding and phosphorylation of Sam68 by Src. *Oncogene.* 18, 4647-53
64. Thomas GM, Frame S, Goedert M, Nathke I, Polakis P, Cohen P. 1999 A GSK3- binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of axin and beta-catenin. *FEBS Lett.* 458, 247-51.
65. Peifer M, Polakis P. 2000 Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. *Science* 287,1606-9.
66. Polakis P. 2000 Wnt signaling and cancer. *Genes Dev*;14, 1837-1851.
67. Spink KE, Polakis P, Weis WI 2000 Structural basis of the Axin-adenomatous polyposis coli interaction. *EMBO J* 19, 2270-2279.
68. Szeto, W., Jiang, W., Tice, D.A., Rubinfeld, B., Hollingshead, P.G., Fong, S.E., Dugger, D.L., Pham, T., Yansura, D.E., Wong, T.A., Grimaldi, J.C., Corpuz, R.T., Singh J.S., Frantz, G.D., Devaux, B., Crowley, C.W., Schwall, R.H., Eberhard, D.A., Rastelli, L., Polakis, P. and Pennica, D. 2001 Overexpression of the Retinoic Acid-Responsive Gene Stra6 in Human Cancers and its Synergistic Induction by Wnt-1 and Retinoic Acid. *Cancer Res* 61, 4197-4204.
69. Rubinfeld B, Tice DA, Polakis P. 2001 Axin dependent phosphorylation of the adenomatous polyposis coli protein mediated by casein kinase 1 epsilon. *J Biol Chem* 276, 39037-39045.
70. Polakis P. 2001 More than one way to skin a catenin. *Cell* 2001 105, 563-566.
71. Tice DA, Soloviev I, Polakis P. 2002 Activation of the Wnt Pathway Interferes with Serum Response Element-driven Transcription of Immediate Early Genes. *J Biol. Chem.* 277, 6118-6123.
72. Tice DA, Szeto W, Soloviev I, Rubinfeld B, Fong SE, Dugger DL, Winer J,

Williams PM, Wieand D, Smith V, Schwall RH, Pennica D, Polakis P. 2002 Synergistic activation of tumor antigens by wnt-1 signaling and retinoic acid revealed by gene expression profiling. *J Biol Chem.* 277,14329-14335.

73. Polakis, P. 2002 Casein kinase I: A wnt'er of disconnect. *Curr. Biol.* 12, R499.

74. Mao, W., Luis, E., Ross, S., Silva, J., Tan, C., Crowley, C., Chui, C., Franz, G., Senter, P., Koeppen, H., Polakis, P. 2004 EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer. *Cancer Res.* 64, 781-788.

75. Shibamoto, S., Winer, J., Williams, M., Polakis, P. 2003 A Blockade in Wnt signaling is activated following the differentiation of F9 teratocarcinoma cells. *Exp. Cell Res.* 292 11-20.

76. Zhang Y, Eberhard DA, Frantz GD, Dowd P, Wu TD, Zhou Y, Watanabe C, Luoh SM, Polakis P, Hillan KJ, Wood WI, Zhang Z. 2004 GEPIS—quantitative gene expression profiling in normal and cancer tissues. *Bioinformatics*, April 8

**EXHIBIT B**

	tumor mRNA	tumor IHC
UNQ2525	+	+
UNQ2378	+	+
UNQ972	+	-
UNQ97671	+	+
UNQ2964	+	+
UNQ323	+	+
UNQ1655	+	+
UNQ2333	+	+
UNQ9638	+	+
UNQ8209	+	+
UNQ6507	+	+
UNQ8196	+	+
UNQ9109	+	+
UNQ100	+	+
UNQ178	+	+
UNQ1477	+	+
UNQ1839	+	+
UNQ2079	+	+
UNQ8782	+	+
UNQ9646	+	-
UNQ111	+	+
UNQ3079	+	+
UNQ8175	+	+
UNQ9509	+	+
UNQ10978	+	-
UNQ2103	+	+
UNQ1563	+	+
UNQ16188	+	+
UNQ13589	+	+
UNQ1078	+	+
UNQ879	+	+



### EXHIBIT 3

143: Mol Cell Proteomics. 2002 Jan;1(1):37-45.

Related Articles, Links

FREE full-text article at  
[www.mcponline.org](http://www.mcponline.org)

#### **Genome-wide study of gene copy numbers, transcripts, and protein levels in pairs of non-invasive and invasive human transitional cell carcinomas.**

**Orntoft TE, Thykjaer T, Waldman FM, Wolf H, Celis JE.**

Department of Clinical Biochemistry, Molecular Diagnostic Laboratory, Aarhus University Hospital, Skejby, DK-8200 Aarhus N, Denmark. [orntoft@kba.sks.au.dk](mailto:orntoft@kba.sks.au.dk)

Gain and loss of chromosomal material is characteristic of bladder cancer, as well as malignant transformation in general. The consequences of these changes at both the transcription and translation levels is at present unknown partly because of technical limitations. Here we have attempted to address this question in pairs of non-invasive and invasive human bladder tumors using a combination of technology that included comparative genomic hybridization, high density oligonucleotide array-based monitoring of transcript levels (5600 genes), and high resolution two-dimensional gel electrophoresis. The results showed that there is a gene dosage effect that in some cases superimposes on other regulatory mechanisms. This effect depended ( $p < 0.015$ ) on the magnitude of the comparative genomic hybridization change. In general (18 of 23 cases), chromosomal areas with more than 2-fold gain of DNA showed a corresponding increase in mRNA transcripts. Areas with loss of DNA, on the other hand, showed either reduced or unaltered transcript levels. Because most proteins resolved by two-dimensional gels are unknown it was only possible to compare mRNA and protein alterations in relatively few cases of well focused abundant proteins. With few exceptions we found a good correlation ( $p < 0.005$ ) between transcript alterations and protein levels. The implications, as well as limitations, of the approach are discussed.

PMID: 12096139 [PubMed - indexed for MEDLINE]

## EXHIBIT 4

214: Urol Res. 2000 Oct;28(5):308-15.

Related Articles, Links



### **Expression of cadherins and catenins in paired tumor and non-neoplastic primary prostate cultures and corresponding prostatectomy specimens.**

**Wang J, Krill D, Torbenson M, Wang Q, Bisceglia M, Stoner J, Thomas A, DeFlavia P, Dhir R, Becich MJ.**

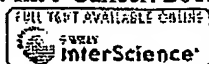
Department of Pathology, University of Pittsburgh Medical Center, PA, USA.

Cadherins are a family of transmembrane proteins that play a crucial role in cell differentiation, cell migration, and intercellular adhesion. Cadherins are associated with catenins through their highly conserved cytoplasmic domain. Down-regulation of E-cadherin protein has been shown in various human cancers. This study examined the expression of cadherins and associated catenins at the mRNA level. Paired tumor and nonneoplastic primary prostate cultures were obtained from surgical specimens. Quantitative multiplex fluorescence reverse transcriptase-polymerase chain reaction (QMF RT-PCR) and quantitative analysis were performed and correlated with immunostain results. Six of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of alpha-catenin and beta-catenin mRNA were also observed. The results of QMF RT-PCR showed good correlation with the results of immunohistochemical studies based on corresponding formalin-fixed sections. In conclusion, this paper presents a coordinated down-regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied. This down-regulation may play an important role in the pathogenesis of prostate cancer.

PMID: 11127708 [PubMed - indexed for MEDLINE]

93: Int J Cancer. 2003 Oct 10;106(6):848-55.

Related Articles, Links



**Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas.**

Munaut C, Noel A, Hougrand O, Foidart JM, Boniver J, Deprez M.

Laboratory of Tumour and Development Biology, University of Liege, Liege, Belgium.

Vascular endothelial growth factor (VEGF) is the major endothelial mitogen in central nervous system neoplasms and it is expressed in 64-95% of glioblastomas (GBMs). Tumour cells are the main source of VEGF in GBMs whereas VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. Infiltrating tumour cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). Recent studies have shown that VEGF expression and bioavailability can be modulated by MMPs. We reported previously that the expression of MT1-MMP in human breast cancer cells was associated with an enhanced VEGF expression. We used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels; activated forms of MMP-2 and MMP-9 were present in 8/18 and 7/18 of GBMs. A majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with strong correlation between VEGF and MT1-MMP gene expression levels, and double immunostaining showed that VEGF and MT1-MMP peptides co-localize in tumour and endothelial cells. Our results suggest that the interplay between metalloproteinases and VEGF previously described in experimental tumours may also be operative in human GBMs. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central importance in the growth of GBMs and represent an interesting target for anti-cancer treatments. Copyright 2003 Wiley-Liss, Inc.

PMID: 12918061 [PubMed - indexed for MEDLINE]

## EXHIBIT 6

89: Leuk Lymphoma. 2003 Aug;44(8):1385-94.

Related Articles, Links

### **Real-time quantitative RT-PCR of cyclin D1 mRNA in mantle cell lymphoma: comparison with FISH and immunohistochemistry.**

**Hui P, Howe JG, Crouch J, Nimmakayalu M, Oumsivah MB, Tallini G, Flynn SD, Smith BR.**

Department of Laboratory Medicine, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208035, New Haven, CT 06520-8035, USA.

Presence of the balanced translocation t(11;14)(q13;q32) and the consequent overexpression of cyclin D1 found in mantle cell lymphoma (MCL) has been shown to be of important diagnostic value. Although many molecular and immunohistochemical approaches have been applied to analyze cyclin D1 status, correlative studies to compare different methods for the diagnosis of MCL are lacking. In this study, we examined 39 archived paraffin specimens from patients diagnosed with a variety of lymphoproliferative diseases including nine cases meeting morphologic and immunophenotypic criteria for MCL by: (1) real-time quantitative RT-PCR to evaluate cyclin D1 mRNA expression; (2) dual fluorescence in situ hybridization (FISH) to evaluate the t(11;14) translocation in interphase nuclei; and (3) tissue array immunohistochemistry to evaluate the cyclin D1 protein level. Among the nine cases of possible MCL, seven cases showed overexpression of cyclin D1 mRNA (cyclin D1 positive MCL) and two cases showed no cyclin D1 mRNA increase (cyclin D1 negative "MCL-like"). In six of seven cyclin D1 positive cases, the t(11;14) translocation was demonstrated by FISH analysis; in one case FISH was unsuccessful. Six of the seven cyclin D1 mRNA overexpressing cases showed increased cyclin D1 protein on tissue array immunohistochemistry; one was technically suboptimal. Among the two cyclin D1 negative MCL-like cases, FISH confirmed the absence of the t(11;14) translocation in both cases. All other lymphoproliferative diseases studied were found to have low or no cyclin D1 mRNA expression and were easily distinguishable from the cyclin D1 overexpressing MCLs by all three techniques. In addition, to confirming the need to assess cyclin D1 status, as well as, morphology and immunophenotyping to establish the diagnosis of MCL, this study demonstrates good correlation and comparability between measure of cyclin D1 mRNA, the 11;14 translocation and cyclin D1 protein.

#### Publication Types:

- Evaluation Studies

PMID: 12952233 [PubMed - indexed for MEDLINE]

## EXHIBIT 7

8: Int J Biochem Cell Biol. 2005 Oct;37(10):2196-206. Epub 2004 Dec 7.

Related Articles,  
Links



### **Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss.**

**Khal J, Hine AV, Fearon KC, Dejong CH, Tisdale MJ.**

Pharmaceutical Sciences Research Institute, Aston University, Birmingham B4 7ET, UK.

Atrophy of skeletal muscle is common in patients with cancer and results in increased morbidity and mortality. In order to design effective therapy the mechanism by which this occurs needs to be elucidated. Most studies suggest that the ubiquitin-proteasome proteolytic pathway is most important in intracellular proteolysis, although there have been no reports on the activity of this pathway in patients with different extents of weight loss. In this report the expression of the ubiquitin-proteasome pathway in rectus abdominis muscle has been determined in cancer patients with weight loss of 0-34% using a competitive reverse transcriptase polymerase chain reaction to measure expression of mRNA for proteasome subunits C2 and C5, while protein expression has been determined by western blotting. Overall, both C2 and C5 gene expression was increased by about three-fold in skeletal muscle of cachectic cancer patients (average weight loss 14.5 $\pm$ 2.5%), compared with that in patients without weight loss, with or without cancer. The level of gene expression was dependent on the amount of weight loss, increasing maximally for both proteasome subunits in patients with weight loss of 12-19%. Further increases in weight loss reduced expression of mRNA for both proteasome subunits, although it was still elevated in comparison with patients with no weight loss. There was no evidence for an increase in expression at weight losses less than 10%. There was a good correlation between expression of proteasome 20S $\alpha$  subunits, detected by western blotting, and C2 and C5 mRNA, showing that increased gene expression resulted in increased protein synthesis. Expression of the ubiquitin conjugating enzyme, E2(14k), with weight loss followed a similar pattern to that of proteasome subunits. These results suggest variations in the expression of key components of the ubiquitin-proteasome pathway with weight loss of cancer patients, and suggest that another mechanism of protein degradation must be operative for patients with weight loss less than 10%.

PMID: 16125116 [PubMed - in process]

269: Am J Pathol. 1999 Sep;155(3):815-22.

Related Articles, Links



**Id-1 and Id-2 are overexpressed in pancreatic cancer and in dysplastic lesions in chronic pancreatitis.**

**Maruyama H, Kleeff J, Wildi S, Friess H, Buchler MW, Israel MA, Korc M.**

Division of Endocrinology, Department of Medicine, University of California, Irvine, USA.

Id proteins antagonize basic helix-loop-helix proteins, inhibit differentiation, and enhance cell proliferation. In this study we compared the expression of Id-1, Id-2, and Id-3 in the normal pancreas, in pancreatic cancer, and in chronic pancreatitis (CP). Northern blot analysis demonstrated that all three Id mRNA species were expressed at high levels in pancreatic cancer samples by comparison with normal or CP samples. Pancreatic cancer cell lines frequently coexpressed all three Ids, exhibiting a good correlation between Id mRNA and protein levels, as determined by immunoblotting with highly specific anti-Id antibodies. Immunohistochemistry using these antibodies demonstrated the presence of faint Id-1 and Id-2 immunostaining in pancreatic ductal cells in the normal pancreas, whereas Id-3 immunoreactivity ranged from weak to strong. In the cancer tissues, many of the cancer cells exhibited abundant Id-1, Id-2, and Id-3 immunoreactivity. Scoring on the basis of percentage of positive cells and intensity of immunostaining indicated that Id-1 and Id-2 were increased significantly in the cancer cells by comparison with the respective controls. Mild to moderate Id immunoreactivity was also seen in the ductal cells in the CP-like areas adjacent to these cells and in the ductal cells of small and interlobular ducts in CP. In contrast, in dysplastic and atypical papillary ducts in CP, Id-1 and Id-2 immunoreactivity was as significantly elevated as in the cancer cells. These findings suggest that increased Id expression may be associated with enhanced proliferative potential of pancreatic cancer cells and of proliferating or dysplastic ductal cells in CP.

PMID: 10487839 [PubMed - indexed for MEDLINE]

## EXHIBIT 9

283: Neurosci Lett. 1999 Apr 23;265(3):191-4.

[Related Articles](#), [Links](#)

ELSEVIER

### **Alterations in neuropeptide Y levels and Y1 binding sites in the Flinders Sensitive Line rats, a genetic animal model of depression.**

**Caberlotto L, Jimenez P, Overstreet DH, Hurd YL, Mathe AA, Fuxe K.**

Department of Neuroscience, Karolinska Institute, Stockholm, Sweden.

Previously, we observed specific alterations of neuropeptide Y (NPY) and Y1 receptor mRNA expression in discrete regions of the Flinders Sensitive Line rats (FSL), an animal model of depression. In order to clarify the correlation between mRNA expression and protein content, radioimmunoassay and receptor autoradiography were currently performed. In the FSL rats, NPY-like immunoreactivity (NPY-LI) was decreased in the hippocampal CA region, while Y1 binding sites were increased; NPY-LI was increased in the arcuate nucleus. Fluoxetine treatment elevated NPY-LI in the arcuate and anterior cingulate cortex and increased Y1 binding sites in the medial amygdala and occipital cortex in both strains. No differences were found regarding the Y2 binding sites. The results demonstrate a good correlation between NPY peptide and mRNA expression, and sustain the possible involvement of NPY and Y1 receptors in depression.

PMID: 10327163 [PubMed - indexed for MEDLINE]

271: Biol Reprod. 1999 Sep;61(3):776-84.

Related Articles, Links

FREE full text article at  
[www.biolreprod.org](http://www.biolreprod.org)

**Follicle-stimulating hormone receptor and its messenger ribonucleic acid are present in the bovine cervix and can regulate cervical prostanoid synthesis.**

**Mizrachi D, Shemesh M.**

Department of Hormone Research, Kimron Veterinary Institute, Bet Dagan, Israel 50250.

The hypothesis that FSH regulates the bovine cervical prostaglandin E(2) (PGE(2)) synthesis that is known to be associated with cervical relaxation and opening at the time of estrus was investigated. Cervical tissue from pre-estrous/estrous, luteal, and postovulatory cows were examined for 1) the presence of bovine (b) FSH receptor (R) and its corresponding mRNA and 2) the effect of FSH on the PGE(2) regulatory pathway in vitro. The presence of bFSHR mRNA in the cervix (maximal during pre-estrus/estrus) was demonstrated by the expression of a reverse transcription (RT) polymerase chain reaction (PCR) product (384 base pairs) specific for bFSHR mRNA and sequencing. Northern blotting revealed three transcripts (2.5, 3.3, and 3.8 kilobases [kb]) in cervix from pre-estrous/estrous cows. The level of FSHR (75 kDa) was significantly higher ( $p < 0.01$ ) in Western blots of pre-estrous/estrous cervix than in other cervical tissues. There was a good correlation between the 75-kDa protein expression and its corresponding transcript of 2.55 kb throughout the estrous cycle as described by Northern blot analysis as well as RT-PCR. Incubation of FSH (10 ng/ml) with pre-estrous/estrous cervix resulted in a 3-fold increase in the expression of FSHR and a 2-fold increase in both G protein ( $\alpha(s)$ ) and cyclooxygenase II. FSH (5-20 ng/ml) significantly increased ( $p < 0.01$ ) cAMP, inositol phosphate ( $p < 0.01$ ), and PGE(2) ( $p < 0.01$ ) production by pre-estrous/estrous cervix but not by cervix at the other stages. We conclude that bovine cervix at the time of the peripheral plasma FSH peak (pre-estrus/estrus) contains high levels of FSHR and responds to FSH by increasing the PGE(2) production responsible for cervical relaxation at estrus.

PMID: 10456856 [PubMed - indexed for MEDLINE]



232: J Urol. 2000 Sep;164(3 Pt 2):1026-30.

[Related Articles](#), [Links](#)



**The decompensated detrusor III: impact of bladder outlet obstruction on sarcoplasmic endoplasmic reticulum protein and gene expression.**

**Stein R, Gong C, Hutcheson JC, Canning DA, Zderic SA.**

Division of Urology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA.

**PURPOSE:** Regulation of calcium ion homeostasis has a significant role in smooth muscle contractility. The sarcoplasmic endoplasmic reticulum, calcium, magnesium, adenosine triphosphatase (SERCA) is a regulatory ion pump that may have a role in the functional outcome after outlet obstruction. We investigate what correlation if any existed between SERCA protein and gene expression, and the contractile properties in the same bladder. **MATERIALS AND METHODS:** Standardized partial bladder outlet obstructions were created in adult New Zealand white rabbits, which were divided into control, sham operated and obstructed groups. Muscle strip studies subcategorized the obstructed group into compensated (force greater than 50% of control) and decompensated (force less than 50% of control). Microsomal membrane and total RNA fractions were prepared from the same bladder tissue. Membrane proteins were used for Western blot analysis using a SERCA specific monoclonal antibody, and total RNA was assessed with Northern blot analysis. **RESULTS:** The relative intensities of signals for the Western and Northern blots demonstrated a strong correlation between protein and gene expression. Furthermore there was a strong association between the loss of SERCA messenger RNA and protein expression and loss of bladder function. **CONCLUSIONS:** Bladder contractility after outlet obstruction is influenced in part by smooth muscle cell ability to maintain calcium homeostasis via SERCA. The loss of SERCA protein expression is mediated by down-regulation in gene expression in the same bladder. These data suggest that smooth muscle ion pump gene expression is in part mechanically (pressure work) regulated.

PMID: 10958733 [PubMed - indexed for MEDLINE]

- 
- 141: Zhonghua Jie He He Hu Xi Za Zhi. 2002 Jun;25(6):337-40. [Related Articles](#), [Links](#)

**[The pathogenic role of macrophage migration inhibitory factor in acute respiratory distress syndrome]**

[Article in Chinese]

Guo Y, Xie C.

Department of Respiratory Medicine, First Affiliated Hospital of Zhongshan University, Guangzhou 510080 China.

**OBJECTIVE** To investigate the expression and pathogenic role of macrophage migration inhibitory factor( MIF) in human acute respiratory distress syndrome(ARDS)

**METHODS** The serum level of MIF in ARDS patients and normal persons were measured by ELISA method. Peripheral blood mononuclear cell (PBMC) MIF expression was determined by flow- cytometry. The expression of MIF mRNA and protein in the lung tissues were detected by using double immuno histochemistry labeling and in situ hybridization. **RESULTS** The serum level of MIF increased significantly in ARDS patients as compared with normal persons ( $P < 0.01$ ). The percentage of PBMC MIF expression was higher in ARDS patients than in normal controls ( $P < 0.01$ ). In situ hybridization and immunohistochemistry showed undetectable or weak MIF mRNA and protein expression in normal lungs. In contrast, there was marked upregulation of MIF mRNA and protein expression in the ARDS lungs. In ARDS macro phages infiltrated the alveolar space and interstitium, most of which also expressed MIF. Infiltrating macrophages were almost restricted to the areas of severe tissue damage. The MIF expression level showed a strong correlation with the number of infiltrating macrophages. **CONCLUSIONS** The serum level of MIF and PBMC MIF expression increased in ARDS patients with enhanced pulmonary MIF expression and macrophage infiltration, which suggests that MIF plays a pivotal role in the pathogenesis of ARDS.

PMID: 12126556 [PubMed - indexed for MEDLINE]

1: Int J Oncol. 2005 Nov;27(5):1257-63.

Related Articles, Links

**Expression of human telomerase reverse transcriptase gene and protein, and of estrogen and progesterone receptors, in breast tumors: Preliminary data from neo-adjuvant chemotherapy.**

Kammori M, Izumiyama N, Hashimoto M, Nakamura K, Okano T, Kurabayashi R, Naoki H, Honma N, Ogawa T, Kaminishi M, Takubo K.

Division of Breast and Endocrine Surgery, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-8655, Japan. kammori-dis@umin.ac.jp.

Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is very closely associated with telomerase activity. Telomerase has been implicated in cellular immortalization and carcinogenesis. In situ detection of hTERT will aid in determining the localization of telomerase-positive cells. The aim of this study was to detect expression of hTERT mRNA, hTERT protein, estrogen receptor (ER) and progesterone receptor (PR) in paraffin-embedded breast tissue samples and to investigate the relationship between hTERT expression and various clinicopathological parameters in breast tumorigenesis. We used in situ hybridization (ISH) to examine hTERT gene expression, and immunohistochemistry (IHC) to examine expression of hTERT protein, ER and PR, in breast tissues including 64 adenocarcinomas, 2 phyllode tumors and their adjacent normal breast tissues. hTERT gene expression was detected by ISH in 56 (88%) carcinomas, but in neither of the 2 phyllode tumors. hTERT protein expression was detected by IHC in 52 (81%) carcinomas, but in neither of the 2 phyllode tumors. Moreover, ER and PR were expressed in 42 (66%) and 42 (66%) carcinomas, respectively, and in neither of the 2 phyllode tumors. In 4 cases of breast carcinoma that strongly expressed hTERT gene and protein before treatment, neo-adjuvant chemotherapy led to disappearance of gene and protein expression in all cases. There was a strong correlation between detection of hTERT gene expression by ISH and of hTERT protein by ICH in tissue specimens from breast tumors. These results suggest that detection of hTERT protein by ICH can be used to distinguish breast cancers as a potential diagnostic and therapeutic marker.

PMID: 16211220 [PubMed - in process]

266: Mol Cell Biol. 1999 Nov;19(11):7357-68.

Related Articles, Links

FREE full text article at  
mcb.asm.org

FREE full text article  
in PubMed Central

### **A sampling of the yeast proteome.**

**Futcher B, Latter GI, Monardo P, McLaughlin CS, Garrels JI.**

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA.  
futcher@cshl.org

In this study, we examined yeast proteins by two-dimensional (2D) gel electrophoresis and gathered quantitative information from about 1,400 spots. We found that there is an enormous range of protein abundance and, for identified spots, a good correlation between protein abundance, mRNA abundance, and codon bias. For each molecule of well-translated mRNA, there were about 4,000 molecules of protein. The relative abundance of proteins was measured in glucose and ethanol media. Protein turnover was examined and found to be insignificant for abundant proteins. Some phosphoproteins were identified. The behavior of proteins in differential centrifugation experiments was examined. Such experiments with 2D gels can give a global view of the yeast proteome.

PMID: 10523624 [PubMed - indexed for MEDLINE]

304: J Biol Chem. 1998 Aug 14;273(33):21161-8.

Related Articles, Links

FREE full text article at  
[www.jbc.org](http://www.jbc.org)

**Overexpression of a DEAD box protein (DDX1) in neuroblastoma and retinoblastoma cell lines.**

**Godbout R, Packer M, Bie W.**

Department of Oncology, Cross Cancer Institute and University of Alberta, 11560 University Ave., Edmonton, Alberta T6G1Z2, Canada.

The DEAD box gene, DDX1, is a putative RNA helicase that is co-amplified with MYCN in a subset of retinoblastoma (RB) and neuroblastoma (NB) tumors and cell lines. Although gene amplification usually involves hundreds to thousands of kilobase pairs of DNA, a number of studies suggest that co-amplified genes are only overexpressed if they provide a selective advantage to the cells in which they are amplified. Here, we further characterize DDX1 by identifying its putative transcription and translation initiation sites. We analyze DDX1 protein levels in MYCN/DDX1-amplified NB and RB cell lines using polyclonal antibodies specific to DDX1 and show that there is a good correlation with DDX1 gene copy number, DDX1 transcript levels, and DDX1 protein levels in all cell lines studied. DDX1 protein is found in both the nucleus and cytoplasm of DDX1-amplified lines but is localized primarily to the nucleus of nonamplified cells. Our results indicate that DDX1 may be involved in either the formation or progression of a subset of NB and RB tumors and suggest that DDX1 normally plays a role in the metabolism of RNAs located in the nucleus of the cell.

PMID: 9694872 [PubMed - indexed for MEDLINE]

## EXHIBIT 16

150: Virchows Arch. 2002 May;440(5):461-75. Epub 2002 Mar 23. [Related Articles](#), [Links](#)



### **Expression of somatostatin receptor types 1-5 in 81 cases of gastrointestinal and pancreatic endocrine tumors. A correlative immunohistochemical and reverse-transcriptase polymerase chain reaction analysis.**

**Papotti M, Bongiovanni M, Volante M, Allia E, Landolfi S, Helboe L, Schindler M, Cole SL, Bussolati G.**

Department of Biomedical Sciences and Oncology, University of Turin, Via Santena 7, 10126 Turin, Italy. [mauro.papotti@unito.it](mailto:mauro.papotti@unito.it)

Somatostatin receptors (SSTRs) have been extensively mapped in human tumors by means of autoradiography, reverse-transcriptase polymerase chain reaction (RT-PCR), in situ hybridization (ISH) and immunohistochemistry (IHC). We analyzed the SSTR type 1-5 expression by means of RT-PCR and/or IHC in a series of 81 functioning and non-functioning gastroenteropancreatic (GEP) endocrine tumors and related normal tissues. Moreover, we compared the results with clinical, pathological and hormonal features. Forty-six cases (13 intestinal and 33 pancreatic) were studied for SSTR 1-5 expression using RT-PCR, IHC with antibodies to SSTR types 2, 3, 5 and ISH for SSTR2 mRNA. The vast majority of tumors expressed SSTR types 1, 2, 3 and 5, while SSTR4 was detected in a small minority. Due to the good correlation between RT-PCR and IHC data on SSTR types 2, 3, and 5, thirty-five additional GEP endocrine tumors were studied with IHC alone. Pancreatic insulinomas had an heterogeneous SSTR expression, while 100% of somatostatinomas expressed SSTR5 and 100% gastrinomas and glucagonomas expressed SSTR2. Pre-operative biopsy material showed an overlapping immunoreactivity with that of surgical specimens, suggesting that the SSTR status can be detected in the diagnostic work-up. It is concluded that SSTRs 1-5 are heterogeneously expressed in GEP endocrine tumors and that IHC is a reliable tool to detect SSTR types 2, 3 and 5 in surgical and biopsy specimens.

PMID: 12021920 [PubMed - indexed for MEDLINE]

114: Eur J Cancer. 2003 Mar;39(5):691-7.

Related Articles, Links



**Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver.**

**van der Wilt CL, Kroep JR, Loves WJ, Rots MG, Van Groenigen CJ, Kaspers GJ, Peters GJ.**

Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.

Deoxycytidine kinase (dCK) is required for the phosphorylation of several deoxyribonucleoside analogues that are widely employed as chemotherapeutic agents. Examples include cytosine arabinoside (Ara-C) and 2-chlorodeoxyadenosine (CdA) in the treatment of acute myeloid leukaemia (AML) and gemcitabine to treat solid tumours. In this study, expression of dCK mRNA was measured by a competitive template reverse transcriptase polymerase chain reaction (CT RT-PCR) in seven cell lines of different histological origin, 16 childhood and adult AML samples, 10 human liver samples and 11 human liver metastases of colorectal cancer origin. The enzyme activity and protein expression levels of dCK in the cell lines were closely related to the mRNA expression levels ( $r=0.75$ ,  $P=0.026$  and  $r=0.86$ ,  $P=0.007$ ). In AML samples, dCK mRNA expression ranged from 1.16 to 35.25 ( $\times 10^{-3}$ ) dCK/beta-actin. In the cell line panel, the range was 2.97-56.9 ( $\times 10^{-3}$ ) dCK/beta-actin of dCK mRNA expression. The enzyme activity in liver metastases was correlated to dCK mRNA expression ( $r=0.497$ ,  $P=0.05$ ). In the liver samples, these were not correlated. dCK mRNA expression showed only a 36-fold range in liver while a 150-fold range was observed in the liver metastases. In addition, dCK activity and mean mRNA levels were 2.5-fold higher in the metastases than in the liver samples. Since dCK is associated with the sensitivity to deoxynucleoside analogues and because of the good correlation between the different dCK measurements in malignant cells and tumours, the CT-RT PCR assay will be useful in the selection of patients that can be treated with deoxycytidine analogues.

PMID: 12628850 [PubMed - indexed for MEDLINE]

67: Regul Pept. 2004 Feb 15;117(2):127-39.

Related Articles, Links

ELSEVIER  
Full Text Article

### Galanin in pituitary adenomas.

Grenback E, Bjellerup P, Wallerman E, Lundblad L, Anggard A, Ericson K, Aman K, Landry M, Schmidt WE, Hokfelt T, Hulting AL.

Department of Molecular Medicine, Endocrine and Diabetes Unit, Karolinska Hospital, S-17176 Stockholm, Sweden. Eva.Grenback@ks.se

Tumor galanin content was measured in extracts from human pituitary adenomas using a specific RIA method for monitoring human galanin. Twenty-two out of twenty-four tumors contained galanin with notably high levels in corticotroph adenomas, varying levels in clinically inactive tumors, and low levels in GH secreting adenomas. Tumor galanin and ACTH contents were closely correlated in all tumors. In four young patients with microadenomas and highly active Mb Cushing tumor galanin was inversely related to tumor volume. The molecular form of tumor galanin, studied with reverse-phase HPLC, was homogeneous with the majority of tumor galanin coeluting with standard human galanin. In the tumors analysed with in situ hybridization there was a good correlation between galanin peptide levels and galanin mRNA expression. In some tumors galanin mRNA and POMC levels coexisted, in others they were essentially in different cell populations. Levels of plasma galanin-LI were not related to tumor galanin concentration, and galanin levels were in the same range in sinus petrosus close to the pituitary venous drainage as in peripheral blood. Corticotrophin releasing hormone injections in two patients caused ACTH, but no detectable galanin release into sinus petrosus. Our results demonstrate that corticotroph, but not GH adenomas, express high levels of galanin, in addition to ACTH, and that in some tumors both polypeptides are synthesised in the same cell population. However, galanin levels in plasma were not influenced by the tumor galanin content.

PMID: 14700749 [PubMed - indexed for MEDLINE]



50: Blood. 2004 Nov 1;104(9):2936-9. Epub 2004 Jul 8.

Related Articles, Links

Full text article at  
[www.bloodjournal.org](http://www.bloodjournal.org)

**BCL2 protein expression parallels its mRNA level in normal and malignant B cells.**

**Shen Y, Iqbal J, Huang JZ, Zhou G, Chan WC.**

Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, USA.

The regulation of B-cell lymphoma 2 (BCL2) protein expression in germinal center (GC) B cells has been controversial. Previous reports have indicated posttranscriptional regulation plays a dominant role. However, a number of recent studies contradicted these reports. Using real-time polymerase chain reaction (PCR) and Standardized Reverse Transcriptase-PCR (StaRT-PCR), we measured the level of mRNA expression in GC, mantle zone (MNZ), and marginal zone (MGZ) cells from laser capture microdissection. Both quantitative RT-PCR measurements of microdissected GC cells from tonsils showed that GC cells had low expression of BCL2 transcripts commensurate with the low protein expression level. These results are in agreement with microarray studies on fluorescence-activated cell sorter (FACS)-sorted cells and microdissected GC cells. We also examined BCL2 mRNA and protein expression on a series of 30 cases of diffuse large B-cell lymphoma (DLBCL) and found, in general, a good correlation. The results suggested that BCL2 protein expression is regulated at the transcriptional level in normal B cells and in the neoplastic cells in most B-cell lymphoproliferative disorders.

PMID: 15242877 [PubMed - indexed for MEDLINE]

1: Blood. 2005 Dec 15;106(13):4315-21. Epub 2005 Aug 25.

Related Articles, Links

Full text article at  
[www.bloodjournal.org](http://www.bloodjournal.org)

**Cyclin D1-negative mantle cell lymphoma: a clinicopathologic study based on gene expression profiling.**

Fu K, Weisenburger DD, Greiner TC, Dave S, Wright G, Rosenwald A, Chiorazzi M, Iqbal J, Gesk S, Siebert R, De Jong D, Jaffe ES, Wilson WH, Delabie J, Ott G, Dave BJ, Sanger WG, Smith LM, Rimsza L, Braziel RM, Muller-Hermelink HK, Campo E, Gascoyne RD, Staudt LM, Chan WC; Lymphoma/Leukemia Molecular Profiling Project.

Department of Pathology and Microbiology, University of Nebraska Medical Center, 983135 Nebraska Medical Center, Omaha, NE 68198-3135, USA. [kfu@unmc.edu](mailto:kfu@unmc.edu)

Cyclin D1 overexpression is believed to be essential in the pathogenesis of mantle cell lymphoma (MCL). Hence, the existence of cyclin D1-negative MCL has been controversial and difficult to substantiate. Our previous gene expression profiling study identified several cases that lacked cyclin D1 expression, but had a gene expression signature typical of MCL. Herein, we report the clinical, pathologic, and genetic features of 6 cases of cyclin D1-negative MCL. All 6 cases exhibited the characteristic morphologic features and the unique gene expression signature of MCL but lacked the t(11;14)(q13; q32) by fluorescence in situ hybridization (FISH) analysis. The tumor cells also failed to express cyclin D1 protein, but instead expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases). There was good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis. Using interphase FISH, we did not detect chromosomal translocations or amplifications involving CCND2 and CCND3 loci in these cases. Patients with cyclin D1-negative MCL were similar clinically to those with cyclin D1-positive MCL. In conclusion, cases of cyclin D1-negative MCL do exist and are part of the spectrum of MCL. Up-regulation of cyclin D2 or D3 may substitute for cyclin D1 in the pathogenesis of MCL.

PMID: 16123218 [PubMed - indexed for MEDLINE]

3: J Gen Virol. 2005 Oct;86(Pt 10):2769-80.

Related Articles, Links

Full text article at  
[vir.sgmjournals.org](http://vir.sgmjournals.org)

**The alpha(v)beta6 integrin receptor for Foot-and-mouth disease virus is expressed constitutively on the epithelial cells targeted in cattle.**

**Monaghan P, Gold S, Simpson J, Zhang Z, Weinreb PH, Violette SM, Alexandersen S, Jackson T.**

Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Surrey GU24 0NF, UK.

Field strains of Foot-and-mouth disease virus (FMDV) use a number of alpha(v)-integrins as receptors to initiate infection on cultured cells, and integrins are believed to be the receptors used to target epithelial cells in animals. In this study, immunofluorescence confocal microscopy and real-time RT-PCR were used to investigate expression of two of the integrin receptors of FMDV, alpha(v)beta6 and alpha(v)beta3, within various epithelia targeted by this virus in cattle. These studies show that alpha(v)beta6 is expressed constitutively on the surfaces of epithelial cells at sites where infectious lesions occur during a natural infection, but not at sites where lesions are not normally formed. Expression of alpha(v)beta6 protein at these sites showed a good correlation with the relative abundance of beta6 mRNA. In contrast, alpha(v)beta3 protein was only detected at low levels on the vasculature and not on the epithelial cells of any of the tissues investigated. Together, these data suggest that in cattle, alpha(v)beta6, rather than alpha(v)beta3, serves as the major receptor that determines the tropism of FMDV for the epithelia normally targeted by this virus.

PMID: 16186231 [PubMed - in process]

Neuroscience. 2005;136(1):147-60. Epub 2005 Sep 21.

Related Articles, Links

**ELSEVIER**  
**Full Text Article**

**Modulation of the glutamatergic receptors (AMPA and NMDA) and of glutamate vesicular transporter 2 in the rat facial nucleus after axotomy.**

**Eleore L, Vassias I, Vidal PP, de Waele C.**

LNRS (CNRS-Paris V), ESA 7060, Centre Universitaire des Saints-Peres, 45 rue des Saints-Peres, 75270 Paris Cedex 06, France.

Facial nerve axotomy is a good model for studying neuronal plasticity and regeneration in the peripheral nervous system. We investigated in the rat the effect of axotomy on the different subunits of excitatory glutamatergic AMPA (GLuR1-4), NMDA (NR1, NR2A-D) receptors, post-synaptic density 95, vesicular glutamate transporter 2, beta catenin and cadherin. mRNA levels and/or protein production were analyzed 1, 3, 8, 30 and 60 days after facial nerve axotomy by in situ hybridization and immunohistofluorescence. mRNAs coding for the GLuR2-4, NR1, NR2A, B, D subunits of glutamatergic receptors and for post-synaptic density 95, were less abundant after axotomy. The decrease began as early as 1 or 3 days after axotomy; the mRNAs levels were lowest 8 days post-lesion, and returned to normal or near normal 60 days after the lesion. The NR2C subunit mRNAs were not detected in either lesioned or intact facial nuclei.

Immunohistochemistry using specific antibodies against GLuR2-3 subunits and against NR1 confirmed this down-regulation. There was also a large decrease in vesicular glutamate transporter 2 immunostaining in the axotomized facial nuclei at early stages following facial nerve section. In contrast, no decrease of NR2A subunit and of post-synaptic density 95 could be detected at any time following the lesion. beta Catenin and cadherin immunoreactivity pattern changed around the cell body of facial motoneuron by day 3 after axotomy, and then, tends to recover at day post-lesion 60 days. Therefore, our results suggest a high correlation between restoration of nerve/muscle synaptic contact, synaptic structure and function in facial nuclei. To investigate the mechanisms involved in the change of expression of these proteins following axotomy, the facial nerve was perfused with tetrodotoxin for 8 days. The blockade of action potential significantly decreased GLuR2-3, NR1 and NR2A mRNAs in the ipsilateral facial nuclei. Thus, axotomy-induced changes in mRNA abundance seemed to depend partly on disruption of activity.

PMID: 16182453 [PubMed - in process]



**High-level mRNA quantification of proliferation marker pKi-67 is correlated with favorable prognosis in colorectal carcinoma.**

**Ihmann T, Liu J, Schwabe W, Hausler P, Behnke D, Bruch HP, Broll R, Windhovel U, Duchrow M.**

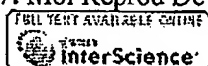
St. Elisabeth Klinik, Klinik für Anesthesiologie, Schmerztherapie und Intensivmedizin, Saarlouis, Germany.

**PURPOSE:** The present study retrospectively examines the expression of pKi-67 mRNA and protein in colorectal carcinoma and their correlation to the outcome of patients. **METHODS:** Immunohistochemistry and quantitative RT-PCR were used to analyze the expression of pKi-67 in 43 archival specimens of patients with curatively resected primary colorectal carcinoma, who were not treated with neo-adjuvant therapy. **RESULTS:** We determined a median pKi-67 (MIB-1) labeling index of 31.3% (range 10.3-66.4%), and a mean mRNA level of 0.1769 (DeltaC(T): range 0.01-0.69); indices and levels did not correlate. High pKi-67 mRNA DeltaC(T) values were associated with a significantly favorable prognosis, while pKi-67 labeling indices were not correlated to prognostic outcome. A multivariate analysis of clinical and biological factors indicated that tumor stage (UICC) and pKi-67 mRNA expression level were independent prognostic factors. **CONCLUSION:** Quantitatively determined pKi-67 mRNA can be a good and new prognostic indicator for primary resected colorectal carcinoma.

**Publication Types:**

- Evaluation Studies

PMID: 15449182 [PubMed - indexed for MEDLINE]



# **c-fos and estrogen receptor gene expression pattern in the rat uterine epithelium during the estrous cycle.**

**Mendoza-Rodriguez CA, Merchant-Larios H, Segura-Valdez ML, Moreno-Mendoza N, Cruz ME, Arteaga-Lopez P, Camacho-Arroyo I, Dominguez R, Cerbon M.**

Facultad de Quimica, Universidad Nacional Autonoma de Mexico, Ciudad Universitaria, Coyoacan 04510, Mexico, D.F., Mexico.

Different studies in ovariectomized estrogen treated animals support the idea that c-fos plays a role in the proliferation of uterine epithelial cells. However, these studies invite us to reassess the role played by c-fos in epithelial cell types of the endometrium during the estrous cycle. The present study was undertaken to determine the c-fos and estrogen receptor (ER) gene expression pattern in the rat uterine epithelium during the estrous cycle in which natural and cyclic changes of steroid hormones occur, and correlate these changes with the proliferation status of this cellular types. Proliferation was assessed during the estrous cycle using bromodeoxyuridine incorporation to DNA. ERalpha and beta proteins were assessed by immunohistochemistry. The regulation of c-fos gene expression in the uterus of intact animals during the estrous cycle was evaluated using both in situ hybridization and immunohistochemistry. Estradiol (E(2)) and progesterone (P(4)) plasma levels were assessed by radioimmunoassay. The results indicated that luminal (LE) and glandular epithelia (GE) presented maximal proliferation during the metestrus (M) and the diestrus (D) days. However, during the proestrus (P) day only LE presented proliferation, and during the estrus (E) day only the stromal cells proliferated. A marked immunostaining for ERalpha was detected in both LE and GE cells during the early phases of the cycle but diminished on the P and the E day. In contrast, ERbeta was undetectable in both epithelia during all stages of the cycle. The highest c-fos mRNA level was detected in both epithelia on the M day, followed by a significant reduction during the other days of the cycle. The highest protein content was observed on the M and D days, and the minimal value was detected on the E day. The c-Fos protein level in LE was increased during M and D days, presenting a high correlation with the cellular proliferation pattern of this cell type. In conclusion, the overall results indicate that c-Fos protein presented a good correlation with uterine epithelial cell proliferation of LE. In the case of GE, the same tendency was observed, although no significant correlation was found. Both in LE and GE, c-fos mRNA did not strictly correlate with its protein levels. c-fos seems to have a posttranscriptional regulation in uterine epithelial cells during the rat's estrous cycle. Copyright 2003 Wiley-Liss, Inc.

PMID: 12589649 [PubMed - indexed for MEDLINE]

FREE full text article at  
clincancerres.aacrjournals.org

**Thymidine kinase, thymidylate synthase, and dihydropyrimidine dehydrogenase profiles of cell lines of the National Cancer Institute's Anticancer Drug Screen.**

**Grem JL, Danenberg KD, Behan K, Parr A, Young L, Danenberg PV, Nguyen D, Drake J, Monks A, Allegra CJ.**

Developmental Therapeutics Department, Medicine Branch, Division of Clinical Sciences, National Cancer Institute at the National Naval Medical Center, Bethesda, Maryland 20889, USA.

**PURPOSE:** To determine the expression of three targets of 5-fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FdUrd) in human tumor cell lines and to compare these with the 50% growth inhibition concentrations (GI(50)) from the National Cancer Institute database. **EXPERIMENTAL DESIGN:** Thymidine kinase (TK) activity was assessed by conversion of [(3)H]thymidine to [(3)H]TMP. Thymidylate synthase (TS) protein expression was determined by Western analysis. TS and dihydropyrimidine dehydrogenase (DPD) mRNA expression were measured by quantitative reverse transcription-PCR. **RESULTS:** The median (range) for the targets were as follows: 5-FU GI(50), 20.8 microM (0.8-536); FdUrd GI(50), 0.75 microM (0.25-237); TK, 0.93 nmol/min/mg (0.16-5.7); in arbitrary units: TS protein, 0.41 (0.05-2.95); TS mRNA, 1.05 (0.12-6.41); and DPD mRNA, 1.09 (0.00-24.4). A moderately strong correlation was noted between 5-FU and FdUrd GI(50)s ( $r = 0.60$ ), whereas a weak-moderate correlation was seen between TS mRNA and protein expression ( $r = 0.45$ ). Neither TS expression nor TK activity correlated with 5-FU or FdUrd GI(50)s, whereas lines with lower DPD expression tended to be more sensitive to 5-FU. Cell lines with faster doubling times and wild-type p53 were significantly more sensitive to 5-FU and FDURD. **CONCLUSIONS:** The lack of correlation may in part be attributable to the influence of downstream factors such as p53, the observation that the more sensitive cell lines with faster doubling times also had higher TS levels, and the standard procedure of the screen that uses a relatively short (48-h) drug exposure.

PMID: 11309351 [PubMed - indexed for MEDLINE]

FREE full text article of  
bio.bmjournals.com

## **Intravitreal invading cells contribute to vitreal cytokine milieu in proliferative vitreoretinopathy.**

**El-Ghrably IA, Dua HS, Orr GM, Fischer D, Tighe PJ.**

Larry A Donoso Laboratory for Eye Research, Department of Ophthalmology, University of Nottingham, UK.

**AIM:** To examine the contribution of infiltrating cells in the local production of cytokines within the vitreous of patients with proliferative vitreoretinopathy (PVR). **METHODS:** The presence of mRNA coding for IL-6, IL-8, IL-1beta, IL-1alpha, TNFalpha, IFNgamma, IL-12, and HPRT was investigated in 25 vitreous samples from patients with PVR, 11 vitreous samples from patients with retinal detachment (RD) not complicated by PVR, and 10 vitreous samples from patients with macular hole (MH). A quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using an internal competitor was used to investigate these samples. From these samples, 15 PVR, 8 RD, and 8 MH were analysed for the protein levels of the same cytokines using enzyme linked immunosorbent assay (ELISA). Spearman correlation was used to test any association between mRNA and cytokine protein levels as an indicator of the contribution these cells make to the intravitreal cytokine milieu. **RESULTS:** A strong correlation was found between mRNA and their respective cytokine levels (protein products) for IL-6, IL-8, IL-1beta, IL-1alpha, TNFalpha, IFNgamma (Spearman  $r = 0.83, 0.73, 0.67, 0.91, 0.73,$  and  $0.73$  respectively), but not for IL-12. The median levels of IL-6, IL-8, IL-1beta, and IFNgamma mRNA and their respective cytokines were significantly higher ( $p < 0.05$ ) in patients with PVR than in those with macular hole. There was no statistically significant difference in the median levels of IL-1alpha mRNA between PVR and MH but the cytokine IL-1alpha was detected at a significantly higher level in PVR compared with MH patients. Between PVR and RD patients, there was no statistically significant difference in mRNA levels for all the investigated cytokines ( $p > 0.05$ ) except for IL-6 where there was a statistical significance ( $p = 0.038$ ). In contrast, the median levels of IL-6, IL-8, and IL-1beta cytokines were significantly higher ( $p < 0.05$ ) in patients with PVR than in those with RD, whereas for IL-1alpha and IFNgamma no significant statistical difference was detected between PVR and RD patients ( $p > 0.05$ ). When results of RD and MH patients were compared, a statistical difference was only detected in mRNA levels of IFNgamma ( $p = 0.008$ ). However, no difference was detected for IFNgamma (protein product) or for any of the other cytokines between RD and MH patients. **CONCLUSION:** Levels of both protein and mRNA encoding IL-6, IL-8, IL-1beta, and IFNgamma is significantly increased in vitreous samples from patients with PVR. The strong correlation between ELISA detectable cytokines (protein products) and their respective mRNA levels suggest that intravitreal, invasive cells are the major source of these cytokines, with the exception of IL-12. Cells invading the vitreous do not appear to locally produce IL-12 mRNA. This would appear to implicate cells peripheral to the



vitreal mass as the major source of this cytokine.

PMID: 11264138 [PubMed - indexed for MEDLINE]



**Human hepatic microsomal epoxide hydrolase: comparative analysis of polymorphic expression.**

**Hassett C, Lin J, Carty CL, Laurenzana EM, Omiecinski CJ.**

Department of Environmental Health, University of Washington, Seattle 98105-6099, USA.

Interindividual variation in the expression of human microsomal epoxide hydrolase (mEH) may be an important risk factor for chemically induced toxicities, including cancer and teratogenesis. In this study, phenotypic variability and mEH genetic polymorphisms were examined in a bank of 40 transplant-quality human liver samples. Immunochemically determined protein content, enzymatic activities, polymorphic amino acids, as well as mEH RNA levels were evaluated in parallel. Enzymatic activity was assessed using (+/-)-benzo[a]pyrene-4,5-epoxide at 2 substrate concentrations. The relative hydrolyzing activities obtained using saturating substrate levels were highly correlated ( $r = 0.85$ ) with results derived from limiting substrate concentrations and exhibit approximately an 8-fold range in activity levels across the panel of 40 liver samples. mEH enzyme activity also demonstrated strong correlation ( $r > \text{or} = 0.74$ ) with an 8.4-fold variation determined for mEH protein content within the same samples. However, these protein/activity measurements were poorly correlated ( $r < \text{or} = 0.23$ ) with mEH RNA levels, which exhibited a 49-fold variation. Two common polymorphic amino acid loci in the mEH protein did not exclusively account for variation in enzymatic activity, although this conclusion is confounded by heterozygosity in the samples. These data demonstrate the extent of hepatic mEH functional variability in well-preserved human tissues and suggest that polymorphism of mEH protein expression is regulated in part by posttranscriptional controls, which may include nonstructural regulatory regions of the mEH transcript.

PMID: 9016823 [PubMed - indexed for MEDLINE]

401: Biochem Biophys Res Commun. 1995 Sep 25;214(3):1009-14.

[Related Articles, Links](#)

**ELSEVIER**  
**EDITORIAL**

**Differential expression of heat shock protein 70 in well healing and chronic human wound tissue.**

**Oberringer M, Baum HP, Jung V, Welter C, Frank J, Kuhlmann M, Mutschler W, Hanselmann RG.**

Department of Traumatology, University of Saarland, Germany.

Heat shock protein 70 (hsp 70) is an important member of the heat shock protein family, which is induced by different forms of stress. We attempted to find out if hsp 70 is also involved in wound healing, which likewise resembles a stress situation for cells too. Therefore we collected tissue samples from well healing and chronic human wound tissue. We used Northern- and Western-blot analysis to study the expression of hsp 70. At the protein level we found a strong correlation between well healing wounds and high expression of hsp 70, whereas chronic wounds showed no or weak expression. Interestingly hsp 70 mRNA did not show this significant correlation, displaying a variant expression pattern in the same kind of wound tissue, possibly due to unknown posttranscriptional regulating step, which has to be investigated in further studies. To localize hsp 70 mRNA and protein was used insitu hybridization and immunohistochemistry. Both displayed an overexpression in endothelial cells of capillary vessels.



**Pre-translational regulation of cytochrome P450 genes is responsible for disease-specific changes of individual P450 enzymes among patients with cirrhosis.**

George J. Liddle C. Murray M. Byth K. Farrell GC.

Department of Gastroenterology and Hepatology, University of Sydney at Westmead Hospital, NSW, Australia.

We have recently reported that disease-specific differential alterations in the hepatic expression of xenobiotic-metabolizing cytochrome P450 (CYP P450) enzymes occur in patients with advanced liver disease. In order to determine whether the observed changes in CYP proteins are modulated at pre- or post-translational levels, we have now examined the hepatic levels of mRNA for CYPs 1A2, 2C9, 2E1 and 3A4 by solution hybridization in the same livers of 20 controls (surgical waste from histologically normal livers), 32 cases of hepatocellular and 18 of cholestatic severe chronic liver disease. CYP1A2 mRNA and CYP1A immunoreactive protein were both reduced in livers with hepatocellular and cholestatic types of cirrhosis. In contrast, CYP3A4 mRNA and protein were reduced only in livers from patients with hepatocellular diseases. For 1A2 and 3A4 there were significant correlations between mRNA species and the respective protein contents ( $r_{S1A2} = 0.74$ ,  $r_{S3A4} = 0.64$ ,  $P < 0.0001$ ). CYP2C9 mRNA was reduced in patients with both cholestatic and hepatocellular types of liver disease, but 2C protein was reduced only in patients with cholestatic dysfunction. The correlation between CYP2C9 mRNA and protein, was also significant ( $r_s = 0.36$ ,  $P < 0.005$ ) but mRNA levels accounted for only 13% of the variability in protein rankings. This is probably a consequence of other CYP2C proteins apart from 2C9 being detected by the anti-2C antibody. CYP2E1 mRNA and protein were reduced in patients with cholestatic liver disease, but in hepatocellular disease the expression of only CYP2E1 mRNA was decreased. CYP2E1 mRNA was significantly correlated with CYP2E1 protein but accounted for only 18% of the variability in protein rankings ( $r_s = 0.43$ ,  $P < 0.0005$ ). Taken collectively these data indicate that the disease-specific alterations of xenobiotic-metabolizing CYP enzymes among patients with cirrhosis is due, at least in part, to pre-translational mechanisms. The lack of a strong correlation between CYP2E1 mRNA and protein suggests that this gene, like its rat orthologue, may be subject to pre-translational as well as translational and/or post-translational regulation.

PMID: 7741759 [PubMed - indexed for MEDLINE]

**Cell localization and regulation of expression of cytochrome P450 1A1 and 2B1 in rat lung after induction with 3-methylcholanthrene using mRNA hybridization and immunohistochemistry.**

Pairon JC, Trabelsi N, Buard A, Fleury-Feith J, Bachelet CM, Poron F, Beaune P, Brochard P, Laurent P.

INSERM Unite 139, Hopital Henri Mondor, Creteil, France.

In order to characterize the response of various pulmonary cell types to polycyclic aromatic hydrocarbons, the expression of cytochrome P450 (CYP) 1A1 and 2B1 mRNA in the lung of rats, with or without induction by 3-methylcholanthrene (3MC), was analyzed by in situ hybridization using appropriate 35S-labeled riboprobes. The expression of the corresponding proteins was investigated immunohistochemically. Following induction with 3MC, the kinetics of mRNA expression differed considerably between Clara cells and type II pneumocytes and venous endothelial cells. In Clara cells, mRNA expression was detected as early as 1 h after induction, peaked between 2 and 4 h, and was completely undetectable at 14 h. In contrast, venous endothelial cells and type II pneumocytes exhibited permanent mRNA expression of CYP 1A1 in 3MC-pretreated rats. These kinetic results explain the striking absence of correlation between mRNA and protein expression observed in Clara cells 24 h after the end of the induction protocol, as these cells exhibited intense protein expression with no mRNA. In contrast, a good correlation was observed for mRNA and protein expression of CYP 2B1, with similar expressions for Clara cells and type II pneumocytes, but no expression in endothelial cells. This study clearly distinguished the regulation of CYP 1A1 expression in the rat lung from that described in the liver. The differences observed in the various lung cell types, whatever the post-transcriptional mechanisms involved, emphasize that studies must be performed at the cellular level in order to understand the specific response to xenobiotics, not only of this organ as a whole but also of its various anatomic structures.

PMID: 7917307 [PubMed - indexed for MEDLINE]

**Cellular location and age-dependent changes of the regulatory subunits of cAMP-dependent protein kinase in rat testis.**

**Landmark BF, Oyen O, Skälhegg BS, Fauske B, Jahnsen T, Hansson V.**

Institute of Medical Biochemistry, University of Oslo, Norway.

This study was undertaken to examine the expression and cellular location of the various cAMP-dependent protein kinase (PKA) subunits in different testicular cell types, using cDNA probes, isoenzyme-specific antibodies and activity measurements. Amounts of mRNA and protein were examined in cultured Sertoli cells, cultured peritubular cells, germ cells (pachytene spermatocytes, round spermatids), Leydig cell tumours as well as whole testes from rats of various ages. In Sertoli cells, there was a good correlation between the amount of mRNA and the respective immunoreactive proteins. In other types of cell, such as germ cells and Leydig tumour cells, this was not always the case. Large amounts of RII beta mRNA were found in Leydig tumour cells, whereas the amount of immunoreactive protein was low. Furthermore, large amounts of small-sized, germ cell-specific mRNAs for RI alpha (1.7 kb) and RII alpha (2.2 kb) were also found in the developing rat testis after 30 to 40 days of age, but the large amounts of mRNA were only partially reflected at the protein level. Pachytene spermatocytes and round spermatids were practically devoid of both RII alpha and RII beta protein. During spermatid differentiation, there was a decrease in RI alpha and an increase in RII alpha protein. Cell specific distribution of the various PKA subunits in testicular cell types is described. In some types of cell, discrepancies between mRNA and protein were demonstrated, which clearly suggest cell specific differences in translational efficiencies for some of these mRNAs, particularly the small-sized mRNAs for RI alpha and RII alpha in meiotic and post-meiotic germ cells.

PMID: 8107013 [PubMed - indexed for MEDLINE]

## Antibodies That React with Predetermined Sites on Proteins

J. Gregor Sütcliffe, Thomas M. Shinnick  
Nicola Green, Richard A. Lerner

Progress in molecular biology and virology has always relied heavily on structural analyses of the components of biological systems. Because of advances in nucleic acid biochemistry during the last decade, culminating with the development of rapid DNA sequencing methods that allow the primary chemical sequences of genes to be read (1), we can now often approach a biological problem by identifying and sequencing the responsible genes rather than analyzing the

corresponding to a putative protein deduced from a string of nucleotides. One solution was to make a bacterium (or yeast) synthesize the novel protein and then raise antibodies to the bacterially synthesized protein. However, experience showed that although it was possible to make bacteria synthesize specific proteins if the genes for these proteins had been isolated, each new gene had to be handled individually and required many manipulations (2). If one were in-

**Summary.** Contrary to previous predictions, relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. Peptides capable of eliciting protein-reactive serums are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins nor to the amino or carboxyl terminals. As such, synthetic peptide immunogens are valuable for eliciting reagents with predetermined specificity that can be used for basic research. In addition, some synthetic peptides are capable of mimicking regions of virus proteins and eliciting immune responses in animals that are protective against the viral agents. Such peptides may thus serve as the basis for safe, chemically defined synthetic vaccines.

relevant proteins. In fact, nucleotide sequence analysis of a new virus or gene is usually a most rewarding initial step, since it provides the information the virus or gene uses to accomplish its phenotype as well as a record of the evolutionary history of that nucleic acid. No fact is more important about a gene than its primary sequence; however, proteins are responsible for the execution of most biological processes.

Given the ease with which it was possible to generate primary sequence data for genes, what was needed was a way to link gene sequences to proteins, particularly when a protein was known only by the sequence of a gene. The obvious answer was to use an antibody, but the problem was how to make an antigen

investigating a "gene" whose product was purely hypothetical, a laborious set of experiments might be necessary to achieve its bacterial expression.

An alternative to biological synthesis of antigens is chemical synthesis. However, most biologically interesting proteins whose sequences can be inferred from genes are in the 15,000 to 150,000 dalton range (15K to 150K), whereas chemical synthesis has practical limits in the 4K to 5K range except in exceptional circumstances. Furthermore, most studies of protein immunogenicity (including the work of Landsteiner, Crumpton, Benjamini *et al.*, Atassi and Suplin, Arnon and Sela and their colleagues, and Cebra and others) (3) indicated that small portions of a protein would, in general, be unlikely to elicit antisera reactive against an intact protein [for a recent review, see (4)]. These studies predicted

that immunogenic sites in small, intact proteins occurred about once every 5K to 10K and that these few loci relied on complex tertiary interactions between amino acid residues near each other in the protein tertiary structure, but distant in relation to the primary linear amino acid structure (so-called conformational determinants) (see Fig. 1). Therefore, it was generally believed that linear sequences in short peptides would not usually mimic these important sites. Recent experiments have challenged this prediction and have shown that small, chemically synthesized fragments of a protein can, in fact, elicit antibodies reactive with the native protein, thus allowing nucleic acid sequences to be parlayed quickly into biological experiments.

### Antibodies to Synthetic Peptides React with Native Proteins

The belief that most antigenic determinants are conformational was first challenged in experiments with chemically synthesized protein fragments from the amino or carboxyl terminals of viral proteins whose sequences had been determined from nucleic acid studies. Antibodies to synthetic peptides [prepared as described in (5, 6)] corresponding to the COOH-terminals of the envelope polypeptide of Moloney murine leukemia virus (MuLV) and the NH<sub>2</sub>- and COOH-terminals of the simian virus 40 (SV40) transforming protein were found to be reactive with the native protein structures (7). That is, each reagent was able to precipitate the corresponding protein from extracts of virus-infected cells. In addition, the fact that antibodies to peptides are specific for predetermined sequences within the intact protein permitted analysis of the precursor of the MuLV envelope polypeptide that undergoes two stages of proteolytic cleavage necessary to generate mature viral proteins (8).

The first reports that protein-reactive antibodies could be elicited by synthetic peptides corresponding to fragments of proteins whose sequences were known only from nucleic acid studies (7) gave a clear indication that a powerful technology was now available (9). The possibility remained, however, that the utility of the technique would be confined to the terminals of proteins where carrier-coupled peptides might mimic their position in the intact molecule. We needed to know whether the technique would be applicable to all proteins and if it would be necessary to predict the naturally anti-

The authors are members of the Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037.

genic regions of proteins in order to select suitable synthetic peptides (10). We used the recently derived sequences of the hepatitis B virus surface antigen (HB<sub>s</sub>Ag) and the hemagglutinin gene of influenza virus type A (HA1) (11) to examine the general utility and expand our understanding of this technology. The two sequences offered different experimental situations. The HB<sub>s</sub>Ag was known to be a molecule whose immunogenicity is critically dependent on its native tertiary structure (12); furthermore, it is extremely hydrophobic. The crystallographic structure of influenza A hemagglutinin had recently been solved (13) and its dominant antigenic sites were well known (14). In addition, since both molecules are the primary targets of neutralizing antibody during natural infection they are of practical interest in dealing with human disease (14-16).

One question we needed to answer concerned the peptide characteristics required to elicit antibodies reactive with the native molecule: Are there restrictions in the chemical makeup or location within the protein structure? The hydrophobic HB<sub>s</sub>Ag glycoprotein consists of 226 amino acid residues. We synthesized 13 peptides using the HB<sub>s</sub>Ag amino acid sequence deduced from the hepatitis B genome sequence as a blueprint (17). These peptides were distributed throughout the primary sequence, but avoided in those regions that showed significant variability in the different viral isolates. Four were subsets of longer peptides. From the data obtained with these peptides we were able to formulate an elementary set of rules for selecting peptides capable of eliciting antisera reactive with native proteins. Peptides that were extremely hydrophobic and those of six or fewer residues were ineffective; longer, soluble peptides, especially those containing proline residues, were effective. Antisera against four of the six HB<sub>s</sub>Ag peptides in the latter category precipitated the HB<sub>s</sub>Ag protein in the viral Dane particles. Precipitation also occurred under conditions approximating physiological (saline solutions), indicating that antibodies to these peptides might be expected to bind antigen *in vivo*. This study showed that linear peptides from more than one region of a protein and, more important, not restricted to its NH<sub>2</sub>- or COOH-terminus could elicit protein-reactive antibodies.

We also needed to know the relation between the sites represented by effective peptides and the antigenic determinants selected by the host in the course of a natural immune response against a virus or a protein. For influenza virus,

those sites immunogenic during natural infection had been mapped by analysis of variants to four domains of the HA1 chain of the hemagglutinin molecule, whose three-dimensional structure was known from x-ray crystallographic studies (13-15). We studied 20 synthetic peptides, many of them overlapping, covering 75 percent of the HA1 primary sequence derived from the nucleotide sequence of a fragment of the influenza genome (6). In accordance with the rules formulated in the HB<sub>s</sub>Ag study (17), these peptides ranged in length from 8 to 39 residues, contained enough polar amino acids to render them soluble and, hence, easy to work with, and usually contained one or more proline residues. Some of the peptides fell within the known antigenic domains of HA1; others were clearly outside these domains. The peptides correspond to regions of the

protein which in the crystal structure appeared as  $\alpha$ -helices,  $\beta$ -sheets, and random coils. Antibodies to 18 of the 20 peptides reacted with HA1 (isolated by bromelain cleavage) or intact virus, demonstrating that sites in proteins accessible to peptide antibodies are more numerous than the few sites recognized in the course of a natural immune response. Furthermore, the information carried within a relatively short linear peptide is sufficient to elicit reactivity against a much larger protein molecule with a complex tertiary and quaternary structure. In more recent studies with peptides selected by the same rules outlined above, 12 of 12 peptides predicted from the MuLV polymerase gene (18) and 18 of 18 peptides from the rabies glycoprotein gene (19) elicited antibodies that precipitated their putative corresponding proteins. Therefore, it appears that, by

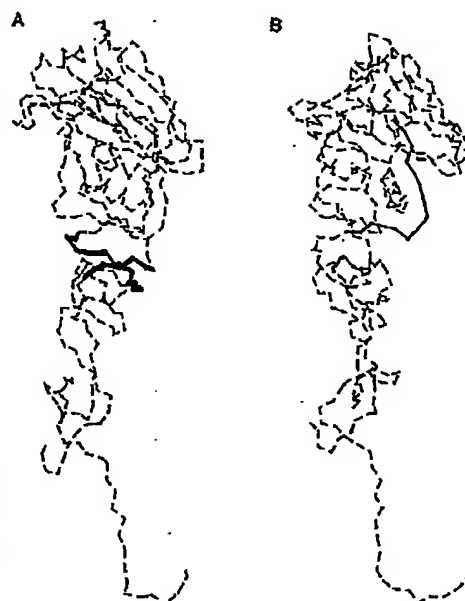


Fig. 1. Simplistic diagrams depicting (A) conformational and (B) sequential (or linear) determinants. The atomic coordinates of the influenza A/Hong Kong HA1 crystallographic structure (13) were rotated until prototypical regions recognized by eye were portrayed with Evans-Sutherland graphics by means of the GRAMPS and GRANNY programs of Donnell and Olson (59) and Connolly (60) to emphasize specific molecular features. The two panels show HA1 at different rotations around its vertical axis. The relevant regions are shown by the solid lines (in bold face in (A)); the rest of the molecule is shown by dashed lines along the  $\alpha$ -carbon backbone. (A) The molecule shows a conformational determinant—two regions of the HA1 polypeptide chain (residues 50 to 55 and 270 to 280) are far apart in the linear protein sequence but intimately related with each other through a disulfide linkage. The chains from the two regions

track each other for several residues. One might expect that an antibody response to this region would probably involve contacts with both peptide chains. This appears to be the case during natural infection; this is HA1 site C of (14). However, antisera to synthetic peptides representing either chain are capable of precipitating HA1 and, in fact, neutralizing the influenza virus. (B) The molecule shows a sequential determinant. The conformation of the peptide chain (residues 255 to 268) in the region shown by the solid line is mostly determined by the particular residues of the chain itself because no other close intramolecular contacts are apparent (nor are there any contacts with HA2 or other HA1 + 2 protomers in the trimeric form of the hemagglutinin). A synthetic peptide might well be expected to antigenically simulate a region such as this. These two extremes are simplifications that help to clarify abstract discussions of protein structure and immunogenicity. In reality, lengthy sequential regions of a protein usually have somewhat extensive contacts with other regions of the protein. However, linear peptides can often mimic the intact protein structure by eliciting a protein-reactive antiserum; therefore, some of the forces that mold global protein structure are already contained in relatively short peptide regions. Clearly, antibody molecules react with a number of spatially related atoms in the antigen molecule, and, in that sense, all determinants are conformational. Recent observations indicate that many peptides from within a protein molecule can act as antigenic determinants; thus many determinants are also sequential. The conformational-sequential nomenclature is probably no longer a useful one.



following simple rules, one can in general select peptides that will elicit antibodies reactive with intact proteins.

In retrospect, it is easy to understand why, in terms of their chemistry, synthetic peptides have been so successful as immunogens in these and several other recent studies and why it was previously believed that they would be ineffective and were therefore not vigorously studied. All of the effective peptides from the influenza HA1 study (6) could be shown, when matched to the crystallographic structure of the protein, to represent regions exposed to the solvent. This may be due, in part, to the fact that they contain polar residues. Polar residues may also provide components of antigenic determinants capable of forming strong electrostatic interactions with antibody. In addition, proline residues may be important because they occur at bends in the peptide chain, often at "corners" exposed to the solution. Because proline residues have an imide rather than an amide bond, several atoms surrounding a proline residue have a fixed three-dimensional relation to one another, whether in a short peptide or in a complete protein structure, and, as such, will be recognized equally by antibody in either situation. Furthermore, residues on the two sides of a proline tend to be near each other because the peptide chain more or less turns back on itself at *cis* proline kinks. This produces a two-chain structure which might be thought of as a minor "conformational" determinant, albeit formed by residues not too far from one another in the linear chemical formula of the protein. Although these explanations for the effectiveness of peptides with certain chemical properties are somewhat speculative, it is now clear that, in solution, peptides and proteins are often similar. Peptides in solution probably attain conformations dictated by their chemical makeup which resemble those that occur in native protein structures. Furthermore, proteins are probably not static structures, always closely resembling their crystalline form; in fact, they may exhibit their various linear domains in many conformations. In support of these notions, Niman and Lerner (20) have shown that 50 percent of the monoclonal antibodies selected for reactivity with one of the peptides in the influenza HA1 study also react with HA1 protein.

In contrast, antisera raised against native HA1 do not react with any of the 20 HA1 peptides (6). Since these peptides span 75 percent of the HA1 primary sequence, including all of the known, mapped determinants, it is clear that

most, if not all, of the immune response against native HA1 is directed against determinants not mimicked by short linear peptides. This observation is consistent with the body of data showing that antigenic determinants on intact proteins are largely conformational (3). Earlier investigators reasoned that because the determinants were conformational, only rarely would a linear peptide be an effective antigen; therefore synthetic peptides would not have general utility. The influenza HA1 study shows that pieces of a protein can elicit antibodies reactive with the whole protein which the whole protein itself cannot elicit (6). Moreover, the studies on HB<sub>Ag</sub>, influenza HA1, MuLV polymerase, and rabies glycoprotein demonstrate that these peptides are not difficult to select (6, 17-19). On the basis of the rules formulated in the HB<sub>Ag</sub> study, one can select one or two peptides from a protein sequence with relatively high confidence of being able to elicit a protein-reactive antiserum, an important economic consideration both in time and research dollars.

#### Protein-Reactive Peptide Antibodies as Reagents for Molecular Studies

Synthetic peptides that elicit reagents capable of reacting with proteins of known primary sequence can be used to establish identity between a protein sequence and the protein itself. Many sequences are of a hypothetical nature, having been deduced from a gene sequence, and an experiment with a synthetic peptide may be more powerful for demonstrating colinearity between a protein and nucleic acid than a series of successful genetic experiments. Antisera to peptides have been used to detect the putative products of the MuLV polymerase (18) and envelope (7) genes as well as the transforming genes of the Moloney sarcoma (21), simian sarcoma (22), feline sarcoma (23), avian myeloblastosis (24), SV40 (7), and polyoma (25) viruses. Such antisera have also been used in similar studies of six human adenovirus 2 (26) transforming genes, the mouse mammary tumor virus long terminal repeat (LTR) (27), and several messenger RNA's from rat brain (28). Peptides from the NH<sub>2</sub>- and COOH-terminals as well as from the middle of the adenovirus transforming proteins elicit protein-reactive sera. Antibodies to peptides have been used to track the processing of polypeptide precursors in cells infected with poliovirus (29), influenza (Fig. 2), and MuLV (8, 18). Because of the predetermined specificity

of the antisera elicited by synthetic peptides, the proteins reacting with them are known to carry the specific peptide sequence. These reagents are particularly powerful when used as sets for tracking simultaneously the fate of various regions of a protein precursor (as shown for influenza HA in Fig. 2 and discussed below for the leukemia virus *pol* product). They are also useful in identifying alternative exon usage [as has been shown with immunoglobulin class D genes (30) and the adenovirus 2 E1A transcription unit (31)]. With the adenovirus 2 E1B transcription unit synthetic peptides have been used to show that the 53K and 19K protein products are translated in different triplet reading frames (26).

When the existence of a protein has been demonstrated, one wants to know its cellular location. Antisera to a peptide from the COOH-terminus of the vesicular stomatitis virus (VSV) G protein do not react with intact infected cells, although polyclonal antisera to the G protein do. When the cells are opened by treatment with detergent, a strong reaction at the inner surface of the membrane is detected with the antiserum to the COOH-terminal peptide (32). These experiments indicate that the VSV G protein spans the plasma membrane with its COOH-terminus protruding into the cytoplasm. In immunofluorescence studies of fixed, transformed fibroblast cells, antisera to a COOH-terminal peptide from the Rous sarcoma virus (RSV) transforming protein were used (33). Fluorescence was codistributed with vinculin at cell-cell contact sites. Antiserum to an interior RSV *src* peptide [which cross-reacts with the endogenous cellular *src* as well as the transforming proteins of Fugu (34) and Y73 (35) viruses] reacted with the focal adhesion plaques of RSV-transformed rat cells (34). Therefore, these reagents, derived by using the sequence of a protein (or gene), are capable of indicating in detail the cellular location of a protein.

Another use for peptide antisera is the correlation of structure with function. If an antiserum to a peptide perturbs an assayable protein function, a protein containing the peptide sequence is implicated. Antiserum to a peptide present in the middle T sequence of polyoma virus inhibits protein kinase function *in vitro* (35), and an antiserum to a peptide present in the feline sarcoma virus *fos* gene sequence also inhibits protein kinase activity (23). Therefore, each of these proteins must have protein kinase activity: the predetermined specificity of the reagent allows one to rule

out the often cited caveats about proteins and enzymatic activities that happen to coincide. Antisera against several peptides in the  $\text{NH}_2$ -terminal half of the putative MuLV *pol* polyprotein inhibit reverse transcriptase activity, whereas some peptides in the COOH-terminal portion inhibit a virion-associated endonuclease activity (18). The two sets of inhibiting antisera immunoprecipitate two different proteins in infected cells, as well as their common precursor. Therefore, not only do the peptide antisera establish the colinearity between the *pol* protein precursor and its nucleic acid sequence, but they identify the products of protein maturation and assign them an enzymatic activity. Antiserum to a COOH-terminal peptide predicted from the poliovirus replicase gene sequence precipitates the core protein p63 and its precursors and inhibits replicase and polyuridylic acid polymerase activities *in vitro*, indicating that these two activities reside in p63 (36). One can thus imagine a range of experiments in which peptide antisera will be used to modify specific behaviors of proteins because they bind to a specific predetermined region of that protein.

When neither function nor structure is known, antisera to peptides can provide a means for purifying the bona fide protein. Walter and his colleagues (37) have coupled to Sepharose the purified immunoglobulin fraction of a peptide antiserum and used this to purify the middle T antigen of polyoma virus. The protein was eluted from the immunoaffinity column by competition with excess peptide. A two-cycle purification scheme in which one uses the immunoglobulin fractions of antisera against two different peptides from a protein sequence might lead to a quite pure protein preparation (37). Immunoprecipitation and gel electrophoresis have been used to isolate a precursor of the MuLV envelope membrane anchor to a purity such that its  $\text{NH}_2$ -terminal sequence could be determined by radiochemical sequencing methods (8). It might eventually be possible to use the complement fixation properties of antigen-antibody complexes as an assay for protein purification after various chromatographic steps (38).

Although most work with synthetic peptides has been done with antisera raised in laboratory animals, some investigators have used clonal populations of peptide-specific lymphocytes for their experiments. The studies of Niman and Lerner (20) on influenza HA1, and those of Gentry *et al.* (34) on the RSV transforming protein, have shown that a high

proportion of hybridomas obtained by fusion of spleen cells from peptide-immunized donors secrete antibody reactive with the native protein. Such doubly specific reagents can be prepared in large quantities and are particularly useful for studies of fixed cells or tissues or for large-scale diagnostic work. Monoclonal populations of B-cell precursors, which can bind peptide but are not yet capable of antibody secretion, have been used to probe the immunological repertoire to determine whether antigen tolerance to host proteins is inherited or acquired (39). In the influenza system, Lamb and his co-workers (40) found that hemagglutinin-specific T-cell populations from human donors responded to 12 of 12 HA1 peptides analyzed. One peptide, corresponding to the COOH-terminal 24 amino acids, appeared to be immunodominant in the sense that three of four T-cell clones were specific for determinants within this sequence.

On the technical side, antibody titer is a relevant concern. Some peptides seem

highly immunogenic, sometimes even without being coupled to carrier protein, eliciting antisera of high titer after a 35-day immunization procedure in which three doses, each of 200 micrograms of peptide, are administered (5, 6). Other peptides seem to require several months and several booster injections to achieve a reasonable titer. A second immunization procedure in which the peptide is coupled to a carrier protein different from that used in the initial series of inoculations seems to be very effective for eliciting a peptide-specific rather than a carrier-specific response (28). For many, but not all, peptides we have examined, glutaraldehyde coupling seems to be more effective than coupling through cysteine residues. However, glutaraldehyde modifies lysine  $\text{NH}_2$  groups and hence can significantly interfere with key amino acids in some peptides. To increase the activity of antisera, and to reduce background activities, purified immunoglobulin can be specifically enriched by adsorption to the antigenic peptide coupled to a solid support (25). The immunoglobulin is then released by a chaotropic agent or removed from the column by competition with excess peptide, then dialyzed in the presence of a chaotropic agent.

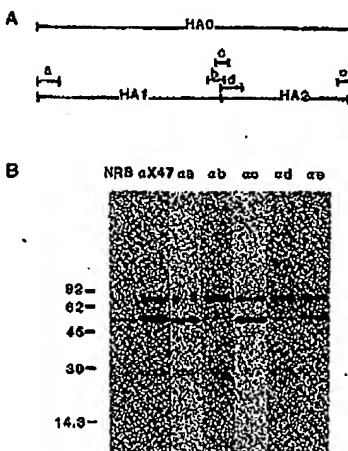


Fig. 2. Illustration of the power of sets of peptide antisera used to track distinct regions of a protein simultaneously. (A) The influenza hemagglutinin precursor (HA0) and eventual products (HA1 and HA2). The synthetic peptides a to e, located within the HA0 sequence as shown ( $\text{NH}_2$ - and COOH-terminal, flanking and spanning the HA1-HA2 junction), were coupled to a carrier and used to immunize rabbits as described (5-7). (B) The resulting antisera (aa, ab, ac, ad, ae) and a positive control serum (antibody to X47 influenza virus, aX47) and a negative control serum (normal rabbit serum, NR5) were used to precipitate extracts of [ $^{35}\text{S}$ ]methionine-labeled influenza X47 virus-infected cells. The five antisera to the peptides and aX47 precipitated the HA0 molecule (this precursor is not appreciably cleaved during infection of cell lines), whereas the other precipitated proteins were nonspecific (sticky) since they also appeared in the normal control lane.

## Synthetic Peptides as Ideal Vaccines

For our studies we have used the immunological targets of infectious pathogens with the goal of eventually applying our findings to the protection of humans and other animals from disease by vaccination with synthetic peptides. Indeed, previous workers have shown that peptides of natural or synthetic origin can elicit antisera capable of neutralizing or binding to virus or bacteriophage *in vitro* [for a review, see (41)]. The coat proteins of tobacco mosaic virus (TMV) (42) and more recently bacteriophage MS2 (43) were fragmented with proteases, and fragments that reacted with neutralizing sera to whole virus were identified and isolated biochemically or chemically synthesized. Antiserum to the natural COOH-terminal six residues of TMV had the ability to bind to the virus *in vitro* and abrogate its infectivity. Antisera to a synthetic MS2 peptide reduced this bacteriophage's infectivity when a secondary antibody to the antibody or "sandwich" reaction was used (no primary neutralization was demonstrated). Similarly, Audibert *et al.* (44) demonstrated that antisera against a synthetic peptide from the diphtheria toxin was capable of

inhibiting toxin activity in vitro. Our studies on HB<sub>s</sub>Ag (17) and influenza HA1 (6) showed that it is possible to select, on the basis of simple chemical properties of regions within viral proteins, peptide immunogens that induce virus-specific antibody. However, these studies did not establish the relation between the capacity to bind virus and the capacity to neutralize infection. What was needed was a demonstration of active protection from a virulent disease in an immunized animal.

In terms of neutralization, there seem to be two classes of virus—those that can be neutralized by antiserum to related viruses and those that are quite subtype-specific in their cross-reactivity. Protective peptides from the first class might cluster in regions whose sequences are conserved across related viruses, whereas protective peptides from the second class may reside in the variable regions. Although it is not necessarily the case that the serum of peptide-protected animals will mimic that of animals protected by classical vaccines, we have proceeded with the thought that reproducing the known serology may be important. The most extensive studies to date have been with four viruses: foot-and-mouth, influenza A, hepatitis B, and rabies.

Seven peptides were chosen (45) from the translated nucleic acid sequence of the type O foot-and-mouth disease virus (FMDV) VP1 protein (46). The VP1 was thought to be a target for neutralizing antibodies, because treatment of this picornavirus with trypsin cleaves only VP1 and this is sufficient to cause the virus particle to lose its immunizing activity (47). Rabbits inoculated with each of the seven peptides made strong antipeptide responses, but antisera to only three of the peptides were capable of neutralizing FMDV in vitro (45). The neutralization was specific to type O virus, as expected from serological considerations. In addition, guinea pigs immunized with a single dose of either of two of the effective peptides (in complete Freund's adjuvant or alum) and challenged with live virus were protected. Thus, a synthetic peptide can elicit a protective immune response. Indeed, one of the peptides that was 20 residues long was as good at protecting the animals from later challenge with 10<sup>4</sup> infectious doses (ID<sub>50</sub>) of FMDV as inactivated virus particles, the classical vaccine (45). Antisera raised in rabbits or cattle against peptides from the equivalent region of other FMDV strains neutralize their appropriate strain of virus. These results are particularly encouraging be-

cause VP1 itself, whether purified from virus or made by genetically engineered bacteria, is a poor immunogen which thus far has not lent itself to the production of a subunit vaccine.

The antipeptide serums to influenza HA1 peptides (6) have been assayed for virus neutralization. Antibodies to 6 of the 20 peptides described in our original structural study neutralized the cytopathic effect of influenza virus on MDCK (canine kidney) cells (48). Subsequently, six more peptides were synthesized filling in the regions missed in the original study, including the HA1-HA2 junction and the other chain of the site C of Wiley *et al.* (14). Antisera to five of these six peptides neutralize the cytopathic effect in vitro (48). In addition, immunizations with several combinations of peptides from the HA1 sequence protect mice from death caused by mouse-adapted influenza virus. However, only two of the five peptides thus far injected individually confer active immunity. Müller *et al.* (49) report neutralization of the cytopathic effect with antiserum to one influenza peptide and inhibition of virus growth in vivo by immunization with that peptide. Although it is too soon to make generalizations about structure, some statements about broad-range neutralization as measured in vitro by Alexander *et al.* (48) are possible. Sequences in the site C region (defined by a disulfide bond between residues 52 and 277 in the native HA1) (14), the COOH-terminal region of HA1 and the HA1-HA2 junction are relatively conserved in the several type A influenza hemagglutinins that have been sequenced. Antisera to peptides from each of these regions neutralize not only viruses of the H<sub>3</sub> subtype (from which the sequence was derived) but also H<sub>1</sub> and B viruses (H<sub>2</sub> has not been tested). Normally, antisera against one subtype of influenza virus do not protect against other subtypes. Thus with peptides it may be possible to construct a vaccine with a broad range of specificity not attainable with the intact protein.

The only relevant model for human hepatitis B is the chimpanzee, so direct studies of protection have not been done; however, extensive serological studies have been conducted in vitro. Prince *et al.* (50) demonstrated that a synthetic peptide corresponding to HB<sub>s</sub>Ag residues 138 to 149 inhibited the binding of hybridomas against the a and d but not the y subdeterminants of HB<sub>s</sub>Ag. In apparently conflicting studies carried out in the chimpanzee, Gerli *et al.* (51) report that a peptide spanning the region 110 to 137 contains the a and y but

not the d specificity (d and y are thought to be allelic). This result is consistent with the results of protein chemistry studies of Peterson *et al.* (52), which show the y/d variation to occur at residues 131 and 134. This is supported by our studies showing that a synthetic peptide corresponding to residues 110 to 137 of the d version elicits d-specific antiserum. Bhatnagar *et al.* (53) have recently reported that a synthetic peptide covering the region 139 to 147 carries the a but not the y/d determinant. The reason for the discrepancy between the results of Prince *et al.* (50) and all of the others is not known, but whichever studies are correct, peptides seem to be able to duplicate serologically important antigenic determinants and elicit immune responses in primates corresponding to those seen during infection. No data on neutralization of the virus or active protection against infection are yet available.

In collaboration with colleagues at the Wistar Institute (19), we have investigated the efficacy of synthetic peptides in protecting animals from rabies virus infection. The purified rabies viral glycoprotein is capable of conferring protection from rabies virus, and all neutralizing sera and hybridomas precipitate this protein (54). Eighteen peptides, representing 56 percent of the primary rabies glycoprotein sequence as deduced from the nucleic acid sequence of its gene (55), were originally synthesized and antibodies were raised against these in rabbits and mice (19). All 18 peptides elicited antisera that bound both rabies glycoprotein and virus as measured in ELISA assays. However, none was capable of neutralizing the virus in vitro or of protecting mice or dogs from challenge from live virus. More recently, it has been shown that the three cyanogen bromide fragments of the glycoprotein capable of eliciting protection were not well covered by the peptide selection (56), hence experiments directed at synthesizing these particular regions are under way. So, while it was easy to pick 18 out of 18 peptides that would elicit binding antibodies, none of these seems capable of protecting against the virus.

Clearly, the studies of FMDV and influenza virus show that one can protect an animal from infection with live virus by injecting synthetic peptides corresponding to parts of the proteins that are the normal targets of neutralizing antibodies. However, the studies with rabies and influenza virus demonstrate that binding antibodies, while easy to elicit, are not necessarily neutralizing antibodies. From the medical-veterinary point of view, one must either resort to synthe-

sizing a series of overlapping peptides covering a complete protein sequence (now a technically feasible, if not particularly elegant, approach) or perform supporting biological experiments to determine which regions of a protein contain its neutralization sites. Although sites capable of interacting with antibody are probably located on the entire surface of the viral protein with frequent representation in its primary linear amino acid sequence, and although such sites can probably be easily mimicked by synthetic peptides, sites susceptible to neutralization seem much less frequent and have not yet been characterized by a set of simple chemical properties. Nonetheless, once the neutralization sites are identified, synthetic peptides seem to be suitable substitutes for whole proteins or viruses (and presumably other infectious agents). For some sites, they may be more effective immunogens than whole protein because they can elicit specificities that whole proteins cannot.

If one projects this technology from prophylaxis to immunological therapy, synthetic peptides from proteins unique to tumor cells may be suitable immunogens for treating neoplasia. The peptides may be from proteins unique to the clonal population of transformed cells or, in tumors of tissues that are dispensable (such as prostate or thyroid), the peptides may be merely tissue-specific, cell-surface markers.

## Conclusions

The desirability of synthetic vaccines of known potency and side effects has been recognized for many years. Some of the drawbacks of currently available inactivated virus vaccines are that they are not stable without refrigeration and sometimes contain incompletely inactivated viruses capable of starting minor epidemics (57). Vaccination with a substance free of any biological contamination introduced in its production (either by virus grown in cell culture or in genetically engineered proteins expressed by *Escherichia coli* protein synthesis factories) and unable to cause any virus-related pathology because of incomplete virus inactivation or imperfect attenuation is the logical goal of protection from infectious disease.

Certainly this technology has not been developed to the level of sophistication that is required for the widespread use of synthetic vaccines in humans and other animals. The adjuvants and carriers used in the studies described in this article are in general much too harsh for human

use, although alum (used in the FMDV study) is suitable. The work of Chedid, Audibert, and Langbeheim and their colleagues (58) may indicate a possible direction for more suitable and ultimately totally defined vaccines. Suitable doses, the possibility of using modified peptides or combinations or polymers of peptides, and the various routes of injection need to be worked out. But a major theoretical obstacle has been overcome in that solving and then synthesizing complex conformational determinants no longer seems necessary.

Now that it has been shown that protection by synthetic peptides is possible, that such peptides can be at least as effective as biological vaccines, that new protein sequences are rapidly being generated as a result of nucleic acid studies, and that the synthetic approach is economically quite feasible (whether it requires the synthesis, by brute force, of all fragments of a protein or directed biochemical-immunological experiments), research to find the best peptides, the best adjuvants, and the best carriers is likely to become an important priority. As the research progresses, and as synthetic vaccines become commonplace, the somewhat tedious processes used today will become streamlined and consequently much more economical.

## References and Notes

1. F. Sanger, *Science* 214, 1205 (1981); W. Gilbert, *ibid.*, p. 1305.
2. E. Yelverton, D. Leung, P. Weck, P. W. Gray, D. V. Goeddel, *Nucleic Acids Res.* 9, 731 (1981); D. G. Kleid, D. Yenzura, B. Small, D. Dawidson, D. Moore, M. J. Grubman, P. D. McKerscher, D. O. Morgan, B. H. Robertson, H. L. Bachrach, *Science* 214, 1125 (1981); F. Charnay, M. Gervais, A. Louis, F. Gilbert, P. Tiollais, *Nature (London)* 286, 893 (1980).
3. K. Landstam, *J. Exp. Med.* 75, 263 (1942); M. J. Crumpton, in *The Antigens*, M. Sela, Ed. (Academic Press, New York, 1974), p. 1; E. Benjamin, J. D. Young, M. Shimizu, C. Y. Leung, *Biochemistry* 3, 1115 (1964); M. Z. Atlas and B. J. Suplin, *ibid.* 7, 688 (1968); R. Arnon, E. Maron, M. Sela, C. B. Anfinsen, *Proc. Natl. Acad. Sci. U.S.A.* 68, 1450 (1971); J. I. Cebra, *J. Immunol.* 86, 205 (1961).
4. R. A. Lerner, *Nature (London)* 259, 592 (1982).
5. In practice, peptides are synthesized according to the high repetitive yield methods developed by Merrifield and his colleagues (A. Marglin and R. B. Merrifield, *Ann. Rev. Biochem.* 39, 841 (1970)) by generally available automatic synthesizers or by hand. They are usually used without further purification, as possible contaminants are either peptides with trivial sequence variations of that designed or they are multimeric forms. The peptides are coupled to a carrier protein (keyhole limpet hemocyanin, edestin, lysoalbumin, and bovine serum albumin have been used) and injected into animals (usually in complete Freund's adjuvant). Some peptides are immunogenic without being coupled to a carrier. Serum from immunized animals is assayed for its ability to bind peptide antigen, either in solution or in a solid phase (enzyme-linked immunosorbent (ELISA)) assay. Positive sera are then tested against native proteins (see (5)).
6. N. Green, R. Alexander, A. Olson, S. Alexander, T. M. Shinnick, J. G. Sutcliffe, R. A. Lerner, *Cell* 28, 477 (1982).
7. J. G. Sutcliffe, T. M. Shinnick, N. Green, F.-T. Liu, H. L. Niman, R. A. Lerner, *Nature (London)* 287, 801 (1982); G. Walter, K. E. Schmidtman, A. Carbone, A. P. Laudano, R. F. Decolli, *Proc. Natl. Acad. Sci. U.S.A.* 77, 5197 (1980).
8. N. Green, T. M. Shinnick, O. Witte, A. Ponticelli, J. G. Sutcliffe, R. A. Lerner, *Proc. Natl. Acad. Sci. U.S.A.* 78, 6023 (1981).
9. R. A. Lerner, J. G. Sutcliffe, T. M. Shinnick, *Cell* 23, 309 (1981).
10. There is nothing intrinsically different between protein sequences derived by translating nucleic acid sequences according to the rules of the genetic code and those empirically determined (except that an empirically determined protein sequence has basis in experiment), but most new protein sequences are determined by nucleic acid sequencing.
11. M. Pasack, T. Goto, W. Gilbert, B. Zink, H. Schaller, P. McKay, G. Leadbetter, K. Murray, *Nature (London)* 282, 575 (1979); W. Minjon, M. Verhoeven, R. Devos, E. Samon, R. Fahg, D. Huybroeck, W. Fiers, G. Threlfall, C. Barber, N. Carey, S. Lottage, *Cell* 19, 683 (1980).
12. G. N. Vyas, K. R. Rao, A. B. Ibrahim, *Science* 178, 1300 (1977).
13. I. A. Wilson, J. I. Skehel, D. C. Wiley, *Nature (London)* 259, 366 (1981).
14. D. C. Wiley, I. A. Wilson, J. I. Skehel, *ibid.*, p. 373.
15. W. G. Laver, O. M. Alf, T. A. Dopheide, C. W. Ward, *ibid.* 283, 459 (1980); R. G. Webster and W. G. Laver, *Virology* 104, 139 (1980).
16. D. L. Peterson, I. M. Roberts, G. N. Vyas, *Proc. Natl. Acad. Sci. U.S.A.* 74, 1530 (1977).
17. R. A. Lerner, N. Green, H. Alexander, F.-T. Liu, J. G. Sutcliffe, T. M. Shinnick, *ibid.* 78, 3403 (1981).
18. T. M. Shinnick and J. G. Sutcliffe, unpublished observations.
19. J. G. Sutcliffe, M. Kleid, T. J. Victor, D. Lopes, H. Koprowski, unpublished observations.
20. H. L. Niman and R. A. Lerner, unpublished observations.
21. J. Papkoff, I. M. Verma, T. Hunter, *Cell* 28, 417 (1982).
22. K. C. Robbins, S. G. Devare, E. P. Reddy, S. A. Aaronson, *Science* 218, 1131 (1982).
23. S. Sen, R. Houghton, C. Scherr, A. Sch, personal communication.
24. J. Lukacs and M. Bafuda, personal communication.
25. G. Walter, M. A. Hutchinson, T. Hunter, W. Eckhart, *Proc. Natl. Acad. Sci. U.S.A.* 78, 4882 (1981).
26. M. Green, K. Brackmann, J. Symington, personal communication; P. B. Branton, S. P. Yee, D. T. Rowe, personal communication.
27. M. Fazel, D. Owen, E. Buell, H. Diggschmann, personal communication.
28. J. G. Sutcliffe, R. I. Mäner, T. M. Shinnick, R. A. Houghton, F. E. Bloom, unpublished observation.
29. M. H. Baron and D. Baltimore, *Cell* 28, 395 (1982); B. L. Semler, C. W. Anderson, R. Hancák, L. F. Dornan, E. Wimmer, *ibid.*, p. 405.
30. T. M. Shinnick and F. Blattner, unpublished observation.
31. L. Feldman and J. Nevins, personal communication.
32. M. C. Williamson, R. Schlegel, I. Pastan, personal communication.
33. E. A. Nigg, B. M. Sefran, T. Hunter, G. Walter, S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* 79, 3322 (1982).
34. L. E. Gentry, L. R. Rohrschneider, J. E. Casnelli, R. Beer, B. G. Krebs, personal communication.
35. B. Schaffhausen, T. L. Benjamin, L. Piko, J. Cannellic, E. Krebs, *J. Biol. Chem.*, in press.
36. M. H. Baron and D. Baltimore, *J. Virol.* 43, 969 (1982).
37. G. Walter, M. A. Hutchinson, T. Hunter, W. Eckhart, *Proc. Natl. Acad. Sci. U.S.A.* 79, 4015 (1982).
38. R. Tjian, *Cold Spring Harbor Symp. Quant. Biol.* 43, 655 (1979).
39. B. Jennings, P. Morrow, N. Klinman, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 41, 420 (1982).
40. J. R. Lamb, D. D. Eckols, P. Lake, J. L. Woody, N. Green, *Nature (London)* 300, 66 (1982).
41. R. Arnon, *Annu. Rev. Microbiol.* 34, 593 (1980).
42. R. A. Anderer, *Biochim. Biophys. Acta* 71, 246 (1983).
43. H. Langbeheim, R. Arnon, M. Sela, *Proc. Natl. Acad. Sci. U.S.A.* 73, 4336 (1976).
44. R. Audibert, M. Jolivet, L. Chedid, J. R. Alouf, P. Boguet, P. Rivelle, O. Siffert, *Nature (London)* 289, 593 (1981).
45. J. L. Hille, R. A. Houghton, H. Alexander, T. M. Shinnick, J. G. Sutcliffe, R. A. Lerner, D. J. Rowlands, F. Brown, *Nature (London)* 298, 30 (1982).
46. C. Kurz, S. Forst, H. Köpper, K. Strahmaler,

47. H. Schaller, *Nucleic Acids Res.* 9, 1919 (1981).
48. K. Strohmaler, R. Franze, K.-H. Adam, *J. Gen. Virol.* 59, 293 (1982).
49. S. Alexander, H. Alexander, N. Green, R. A. Lerner, personal communication.
50. G. Müller, M. Shupia, R. Arnon, *Proc. Natl. Acad. Sci. U.S.A.* 79, 369 (1982).
51. A. M. Prince, H. Ikrom, T. P. Hopp, *ibid.*, p. 579.
52. J. L. Gerin *et al.*, *ibid.*, in press.
53. D. L. Peterson, N. Natsu, F. Gavilanes, *J. Biol. Chem.* 257, 10414 (1982).
54. P. K. Bhatnagar, B. Papas, H. E. Blum, D. R. Millich, D. Nitecki, M. J. Karcis, G. N. Vyas, *Proc. Natl. Acad. Sci. U.S.A.* 79, 4400 (1982).
55. J. H. Cox, B. Deitzschold, L. G. Schneider, *Infect. Immun.* 16, 743 (1977).
56. A. Anlidons, W. H. Wunner, P. J. Curtis, *Nature (London)* 294, 275 (1981).
57. B. Deitzschold, T. J. Wistor, R. MacFarlan, A. Varrichio, *J. Virol.*, in press.
58. J. Beale, *Nature (London)* 298, 14 (1982); A. M. Q. King, B. O. Underwood, D. McCahon, J. W. I. Newman, F. Brown, *ibid.* 293, 479 (1981).
59. L. Chedid, F. Audibert, A. Johnson, *Prog. Allergy* 25, 63 (1978); H. Langbeheim, R. Arnon, M. Seia, *Immunology* 35, 573 (1978); F. Audibert, M. Jolivet, L. Chedid, R. Arnon, M. Seia, *Proc. Natl. Acad. Sci. U.S.A.* 79, 5042 (1982).
60. T. J. O'Donnell and A. J. Olson, *Computer Graphics* 15, 133 (1981).
61. M. Connolly, unpublished results.
62. We acknowledge the efforts of our co-workers who contributed to designing the experiments and collecting the data to which we refer herein, and thank many of our colleagues for sharing their data in advance of publication. We also thank A. Olson for producing Fig. 1 and R. Ogata for comments on the manuscript. Portions of this work were supported by grants from the American Cancer Society (NP-359) and the National Institutes of Health (R01 AI 18509). This is paper No. 2824 of the Research Institute of Scripps Clinic.

## Protein Engineering

Kevin M. Ulmer

In the last decade, genetic engineering technology has been developed to the point where we can now clone the gene for essentially any protein found in nature. By precise manipulation of the appropriate regulatory signals we can then produce significant quantities of that protein in bacteria. Recent advances in chemical synthesis of DNA now permit virtually unlimited genetic modification, and offer the prospect for developing protein engineering technology to create novel proteins not found in nature. By starting with the known crystal structure for a protein we would like to directly modify the gene to alter that structure in a predictable fashion, targeted to improve some functional property. At each stage we could verify the structural and functional changes that actually occurred and thereby refine and extend our predictive capability. Step by step, as we gain facility with this technique and learn the detailed rules that relate structure and function, we should be able to create proteins with novel properties which could not be achieved as effectively by any other method.

### Rationale

Despite the fact that biochemists have characterized several thousand enzymes, there are only a handful that could be considered enzymes of commerce. Indeed, only a dozen enzymes have worldwide sales in excess of \$10 million per year, and together they ac-

count for more than 90 percent of the total enzyme market (1). Frequently the limiting factor in the industrial use of an enzyme has simply been the high cost of isolating and purifying adequate amounts of the protein. Part of the solution to this problem lies with the ability of genetic engineers to greatly amplify the produc-

**Summary.** The prospects for protein engineering, including the roles of x-ray crystallography, chemical synthesis of DNA, and computer modeling of protein structure and folding, are discussed. It is now possible to attempt to modify many different properties of proteins by combining information on crystal structure and protein chemistry with artificial gene synthesis. Such techniques offer the potential for altering protein structure and function in ways not possible by any other method.

tion of specific enzymes in microorganisms, but beyond cost there are often other limitations to the broader use of enzymes which stem from the fact that the desired industrial application is far removed from the physiological role normally played by the enzyme. In particular, industrial applications require generally robust enzymes with a long half-life under process conditions. Frequently the desired substrate or product is somewhat different from the physiological one, and often the chemical conditions for the reaction are decidedly nonphysiological, ranging to extremes of pH, temperature, and concentration. If enzymes are to be more widely used as industrial catalysts, we must develop methods to tailor their properties to the process of interest. The list of properties of enzymes we would like to be able to con-

trol in a predictable fashion would include the following:

- 1) Kinetic properties including the turnover number of the enzyme and the Michaelis constant,  $K_m$ , for a particular substrate.
- 2) Thermostability and temperature optimum.
- 3) Stability and activity in nonaqueous solvents.
- 4) Substrate and reaction specificity.
- 5) Cofactor requirements.
- 6) pH optimum.
- 7) Protease resistance.
- 8) Allosteric regulation.
- 9) Molecular weight and subunit structure.

The solutions to these problems have included extensive searches for the best suited naturally occurring enzyme, mu-

tation and selection programs to enhance the native enzyme's properties, and chemical modification and immobilization to obtain a stable and functional biocatalyst. From such work we know that all of these properties can in general be improved. Specific examples of what has been achieved by these methods and how protein engineering can build on this knowledge to yield still further improvements are cited below.

It is not uncommon to observe wide variations in properties such as turnover number,  $K_m$ , molecular weight, temperature optimum, thermostability, pH optimum, and pH stability among enzymes of the same type isolated from different

Kevin M. Ulmer is director of exploratory research at Genex Corporation, Science and Technology Center, 16020 Industrial Drive, Gaithersburg, Maryland 20877.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: \_\_\_\_\_**

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**